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Jessica F. Andrade for the degree of Master of Science in Fisheries Science presented on March 10, 2017.

Title: Effects of Elevated CO_2 on Speckled Sanddab (*Citharichthys stigmaeus*) Behavior

Abstract approved:

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Thomas P. Hurst

The direct and indirect effects of ocean acidification (OA) are a growing concern, particularly in areas already experiencing elevated levels of oceanic CO_2. Studies with marine fishes suggest that elevated CO_2 levels may affect behavior by interfering with an important brain neurotransmitter. Studies examining the effects of OA fish behavior have been predominately conducted on tropical fishes; few have been conducted on fishes from temperate and boreal regions. The productive ecosystems of these regions, such as those of the California Current, support important commercial fisheries. Parts of the California Current are already experiencing elevated CO_2 during seasonal upwelling events. Flatfishes are an important component of the ecosystems of the California Current; not only do flatfishes support important regional fisheries, but they also are an important link in energy transfer within marine food webs. To date there has been little work examining the effects of OA on flatfish behavior. In laboratory experiments, I first examined speckled sanddab (*Citharichthys stigmaeus*) behavioral responses to potential predation cues (predator odor, damaged skin cues from injured conspecifics, and sight of a predator) under ambient CO_2 conditions. Whereas sanddab exhibited reductions in conspicuousness and foraging following exposure to the sight of a predator, they increased activity and foraging following exposure to damaged skin cues from injured conspecifics. I then examined the effects of elevated CO_2 levels on posture, activity, and foraging of sanddab, and if CO_2 altered their responses to damaged skin cues. CO_2 treatments reflected present-day CO_2 levels (~ 400μatm) and those predicted to occur

over the next 150 years (~1,000 μatm and ~1,600 μatm). While there was no major effect of CO_2 treatment on the behavior of speckled sanddab, there were non-significant trends of fish from the medium CO_2 treatment exhibiting the lowest posture and activity scores, longest feeding latencies, and fewest feeding strikes. Results suggest that aspects of speckled sanddab behavior might resistant to OA. It is also possible that prolonged exposure to elevated CO_2 enabled speckled sanddab to compensate, mitigating the effects observed in other fishes following shorter-term exposure to elevated CO_2. Experiments further examining the interactive effects of elevated CO_2 with other environmental conditions on speckled sanddab behavior can also provide insight into the potential ecological consequences of life in an ocean altered by global climate change. Additional studies of ecologically relevant behaviors across diverse species assemblages will be needed to evaluate the impact of ocean acidification on marine food webs.

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Effects of Elevated CO_2 on Speckled Sanddab (*Citharichthys stigmaeus*) Behavior

by
Jessica F. Andrade

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Jessica F. Andrade, Author

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Chapter 1 – Introduction

Ocean acidification (OA) is a process whereby elevated levels of oceanic carbon dioxide cause a decrease in oceanic pH. A facet of global climate change, OA is an anthropogenic process caused by the increased rate of global carbon consumption that has occurred since the industrial revolution (Gattuso and Hansson 2011). As carbon consumption increases, atmospheric carbon dioxide increasingly dissolves in seawater causing oceanic pH to decrease. Average oceanic pH has already decreased by 0.1 units over the past 250 years and is expected to decrease another 0.2-0.4 units as atmospheric carbon dioxide levels increase from 400 ppm to 800-1,100 ppm by the end of the century (IPCC 2013). This anthropogenically induced increase in rate of CO_2 production is unprecedented and of concern, because elevated CO_2 can have wide-ranging effects on a variety of marine species (Kroeker et al. 2013, Wittmann and Pörtner 2013).

Studies suggest that elevated CO_2 can negatively affect calcification, growth, and survival at different life stages of many calcifying species such as molluscs and echinoderms (Bednaršek et al. 2014, Byrne et al. 2011). Reduced growth and malformed, degraded calcified structures are thought to make these animals more susceptible to predation (Kroeker et al. 2010). However, there is much variation in responses both among and within species as some species have exhibited no deleterious effects to calcification under elevated CO_2 (Comeau et al. 2014). Furthermore, OA-induced reductions in calcification rates might be mitigated if beneficial concurrent environmental conditions such as those that provide food supply adequate to cover the additional energetic expenditure of calcification or osmoregulation (Ramajo et al. 2016). Calcifying

species can also shift energy allocation from an associated trait to compensate for the additional energy costs of calcification. For example, Olympia oysters (*Ostrea lurida*) were able to maintain the same degree of shell thickness when exposed to elevated CO_2 as in ambient seawater by reducing growth, but their smaller size made oysters more vulnerable to predation by an invasive gastropod (Sanford et al. 2014).

Elevated CO_2 exposure can also adversely affect fishes in a variety of ways and at different life history stages. Experiments with larval fish have revealed that elevated CO_2 exposure can reduce growth, development, and survival in some species (Bauman et al. 2012, Frommel et al. 2011). However, other fishes appear more physiologically resilient to elevated CO_2, exhibiting no effect on growth or survival (Munday et al. 2011b, Hurst et al. 2013). Furthermore, elevated CO_2 exposure can increase growth and egg production in some fishes (Munday et al. 2009a, Hurst et al. 2012, Miller et al. 2013); however, these potentially beneficial effects may have high energetic costs that may not be conducive to long-term survival with chronic exposure to high CO_2 levels.

Recent work has suggested that fishes lacking OA-induced changes in growth and development can still exhibit impaired sensory biology and altered behavior (Munday et al. 2009b, Dixson et al. 2010) likely involving an OA-induced impairment in GABA-A, a brain neurotransmitter involved in a variety of behaviors (Nilsson et al. 2012). Moreover, elevated CO_2 has been shown to increase otolith calcification rates (Checkley et al. 2009, Bignami et al. 2013, Réveillac et al. 2015). The change in otolith size is hypothesized to disrupt hearing capabilities and thus potentially alter fish behavioral responses to auditory stimuli (Bignami et al. 2013, Rossi et al. 2016). While causality has not been established,

exposure to elevated CO_2 conditions has elicited both enlarged otoliths and a loss of preference for an auditory settlement cue (Rossi et al. 2016) in larval mulloway (*Argyrosomus japonicus*). Regardless of the OA-induced mechanism(s) altering behaviors, exposure to elevated CO_2 has been found to induce a loss of normal sensory-mediated behaviors to various environmental cues besides settlement cues in a variety of fishes. In some fishes, exposure to elevated CO_2 has been shown to eliminate a preference for olfactory foraging cues (i.e. prey scent) and reduce foraging behaviors (Cripps et al. 2011, Dixson et al. 2014). Exposure to olfactory predation cues (i.e. odor from a predator or damaged skin extract from injured conspecifics) following elevated CO_2 exposure has also been experimentally demonstrated to elicit a loss or complete reversal of antipredator behaviors (Dixson et al. 2010, Munday et al. 2010, Ferrari et al. 2011, Lönnstedt et al. 2013). Failing to recognize and respond to predation risk can increase an individual's predation vulnerability (Munday et al. 2010). However, not all fishes examined have exhibited a change in sensory-mediated responses. For instance, juvenile Atlantic cod (*Gadus morhua*) maintained an avoidance of predator odor under elevated CO_2 conditions (Jutfelt and Hedgärde 2013). Furthermore, antipredator responses to the sight of a predator appear to remain intact in some fishes following OA exposure (Ferrari et al. 2012b, Lönnstedt et al. 2013). Yet those species exhibiting OA-induced changes in behavioral responses to some cues and not to other cues may still be vulnerable to predation, as elevated CO_2 can also prevent fish from learning to recognize predator olfactory cues as threatening (Ferrari et al. 2012a). Thus, OA-induced changes

in behavior and perception of the environment can indirectly affect survival and productivity through altered species interactions and ecological processes.

The potential ecological consequences of altered behavioral responses to olfactory foraging cues or predation cues are self-evident. However, some behavioral traits that are studied in fishes have unknown or ambiguous ecological relevance, and thus the ecological consequences of OA-induced changes in these behaviors are less clear. One such trait is behavioral lateralization, or the propensity of a fish to turn one way over another. In fishes, behavioral lateralization is characterized as the degree of right- or left-turning biases in an individual and is thought to be associated with visual stimuli (Facchin et al. 1999). Behavioral lateralization has been hypothesized to confer both advantages and disadvantages to fishes, but all are highly contextually dependent on factors such as the life history and current environment of a fish. For instance, a pre-programmed escape response in the presence of a solitary predator can make a schooling fish less vulnerable to predation if the fish is able to more-rapidly turn with and thus remain with the group when attacked. However, this same fish can become more vulnerable to predation by remaining with the group if multiple predators are cooperatively feeding. The high variation in the degree of lateralization within and among species further complicates the ecological relevance of this trait. Altered degree of lateralization has been observed in fishes exposed to elevated CO_2 and is used as evidence supporting the mechanism of neuronal impairment as the cause of changes in a variety of behaviors under OA conditions (Domenici et al. 2012, Nilsson et al. 2012). However, the ecological implications of altered behavioral lateralization are unclear as

the ecological relevance of lateralization is ambiguous. Testing known ecologically relevant behaviors under OA conditions is crucial to understanding future population dynamics and developing management strategies to mitigate the consequences of global climate change on marine ecosystems.

The majority of OA behavioral studies to date have been conducted on fishes from tropical environments (Clements and Hunt 2015); however, more recently there have been studies conducted on fishes from temperate and high-latitude regions. Studies examining the effects of OA on fishes of these regions are especially important because evidence suggests that increases in CO_2 levels will occur the fastest in high-latitude waters (Fabry et al. 2009). Furthermore, areas such as the productive California Current are already experiencing seasonal episodic events of elevated CO_2 when waters high in CO_2 are upwelled into coastal regions (Hauri et al. 2013, Feely et al. 2016). It has been hypothesized that fishes experiencing little or no variation in CO_2 levels, such as fishes from coral reef and pelagic environments, might be more susceptible to the effects of OA. Conversely, fishes already experiencing high variation in CO_2 levels such as those in upwelling regions might be more resistant to the effects of elevated CO_2 associated with OA (Munday et al. 2011a). However, experiments to date have yet to find a clear pattern. Flatfishes are an important component of ecosystems already experiencing high variation in CO_2 levels, supporting fisheries and serving as central links within food webs. Experiments have revealed variation in physiological effects of elevated CO_2 on the early life stages of different flatfish species (Chambers et al. 2014, Gräns et al. 2014, Hurst et

al. 2016); however, there has only been one study to date that has examined the effects of OA on the behavior of a flatfish (Sampaio et al. 2016).

Flatfishes have evolved a unique set of life history traits intricately linking their morphology and behavior. During metamorphosis from pelagic larvae to benthic juveniles, flatfishes undergo eye migration and a shift in orientation to swimming on the eyeless, “blind” side. These traits minimize the profiles of flatfish on the sediment thereby reducing conspicuousness and predator detection. Juvenile and adult activity is primarily characterized by saltatory movements along the substrate surface, which further reduces their conspicuousness and facilitates a cryptic lifestyle. A suite of anti-predation-related behaviors has been studied in laboratory experiments (Boersma et al. 2008, Lemke and Ryer 2006a, Lemke and Ryer 2006b) involving several North Pacific juvenile flatfishes, including northern rock sole (*Lepidopsetta polyxystra*), Pacific halibut (*Hippoglossus stenolepis*), and English sole (*Parophrys vetulus*). Although all predominantly rely on inconspicuous behaviors such as reducing activity and burying in the sediment to avoid predation, they exhibit different anti-predation strategies. These experiments primarily relied on visual exposure to a predator; no study to date has examined flatfish behavioral responses to olfactory predation cues. Because the act of foraging involves movement that could attract the attention of a predator (e.g. elevated posture and activity), foraging presents a contrast to flatfish anti-predation behaviors. Thus, their cryptic nature forces flatfishes to allocate time between foraging and anti-predation behaviors in a manner that facilitates maximal growth and survival.

Various stimuli associated with predators and predation events can elicit anti-predation behaviors in fishes (Ferrari et al. 2010, Lönnstedt et al. 2012). Such stimuli include the scent of a predator, sight of a predator, and the scent of damaged skin from injured conspecifics. It stands to reason that the degree to which fish express anti-predation behavioral responses to each of these stimuli would correspond positively to perceived predation risk. If so, in flatfishes, increasing predation risk would translate to decreasing conspicuous behaviors such as posture and activity (Boersma et al. 2008). Of the three aforementioned examples of predation-associated stimuli, it is expected that the scent of a predator would elicit the weakest anti-predation response in juvenile flatfishes as the inability to verify the distance of a predator or whether the predator is actively hunting. Because the scent of injured conspecifics is suggestive of a local, recent predation event, it is thought that exposure to this stimulus would elicit greater anti-predation responses than to that of predator odor. The sight of a predator is expected to elicit the greatest degree of anti-predation behaviors as it confirms the immediate presence of a nearby predator for the duration it remains in view. It is hypothesized that juvenile flatfishes would reduce conspicuousness in a graded manner such that exposure to predator odor, the scent of injured conspecifics, and the sight of a predator would reflect increasing perceived predation risk, eliciting continuously reduced conspicuousness (Figure 1).

Speckled sanddab (*Citharichthys stigmaeus*) is a flatfish species found in nearshore, benthic habitats along the California Current. Juveniles and adults primarily reside along the coast and in estuaries and bays at depths < 40 m (Rackowski and Pikitch

1989). Speckled sanddab feed on epifaunal and infaunal prey such as crustaceans, polychaetes, and bivalve siphons (Wakefield 1984, Rackowski and Pikitch 1989). Although their small size (< 17 cm total length) precludes speckled sanddab from being an important commercial fishery, their abundance within ecosystems and in the diets of seabirds and marine mammals (Elliot et al. 2015; Wright et al. 2007) suggests that speckled sanddab have an important role in energy transfer. However, no study to date has described any aspects of their behavioral ecology.

This study consists of two laboratory experiments examining speckled sanddab activity and foraging behaviors. I first conducted a behavioral characterization experiment to describe speckled sanddab behavioral responses to potential predation cues under ambient CO_2 conditions and identified the cue that elicited the most obviously consistent behaviors. I then tested the behavioral responses to this single predation-associated stimulus under elevated CO_2 conditions during the OA experiment. If sanddab are similar to other fishes exposed to elevated CO_2, it is expected that sanddab would not reduce conspicuousness, but rather would maintain or increase posture and activity. Furthermore, it is believed that regulating internal pH under elevated CO_2 conditions is energetically costly. Thus it is conceivable that elevated CO_2 could cause increases in foraging behaviors such as posture and activity, even in the absence of cues.

Figures

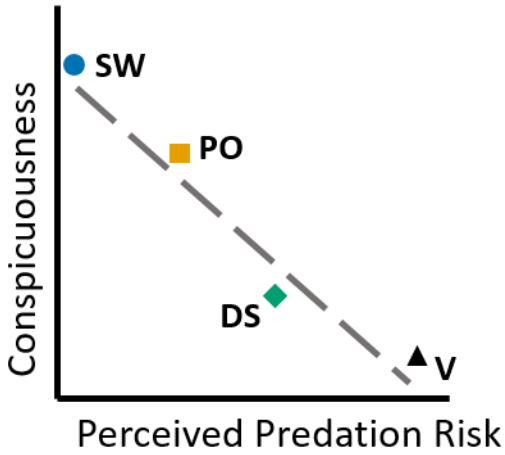

Figure 1. Depiction of hypotheses for the behavioral characterization experiment. Speckled sanddab are hypothesized to continually reduce conspicuous behavioral responses (e.g. posture, activity) to stimuli reflecting increasing perceived predation risk. A seawater blank (SW) served as the cue control. Potential predation cues included predator odor (PO), damaged skin cues (DS), and the sight of a predator (V).

Chapter 2 – Methods

Experimental Overview

This study comprises two experiments, the (1) behavioral characterization and (2) ocean acidification experiments. In the behavioral characterization experiment, I first examined juvenile speckled sanddab baseline behavioral responses to multiple potential predation cues before, during, and after food exposure to inform examination of ecologically relevant behaviors under elevated CO_2 conditions. In the ocean acidification (OA) experiment, I examined the effects of elevated CO_2 concentrations on speckled sanddab behavioral responses to one of these cues.

Collection and Maintenance

Age-0 speckled sanddab were collected in Yaquina Bay, Oregon over the summer of 2014 by the Fisheries Behavioral Ecology Program of the Alaska Fisheries Science Center. Fish were collected at depths of 2-15 m using a 2-m otter trawl with a 3-mm mesh cod end towed at 1-2 knots. Fish were held at Hatfield Marine Science Center in flow-through tanks containing chilled seawater maintained at 7-9°C from Yaquina Bay and were exposed to a 12:12 light:dark cycle. Tanks contained a thin layer of a 1:1 mixture of 0.65-mm and 0.35-mm sand to allow fish to bury. During initial holding, fish were fed a mixture of krill and gelatinized marine protein, amino acids, and vitamins to satiation three times per week.

Fish were transferred to 100-L acclimation tanks prior to experimentation. In these tanks, groups of four fish were acclimated to a remote food delivery method similar

to that used in experimental trials (and to CO_2 treatments for the OA experiment). Three times per week, food was delivered throughout the day using belt feeders. A length of vertical tubing extending from the edge of the belt feeder to the middle of the water column delivered food below the seawater surface. This method delivered food at unpredictable times and was used to remove the association of food with external stimuli (e.g. experimenter presence) and trained fish to become more vigilant and therefore more consistent in detecting and taking advantage of episodic food availability. To standardize the amount of food given to fish, diet was switched to BioDiet® pellets. Belt feeders delivered a combination of four to eight 3-mm and eight to sixteen 2-mm pellets to each tank over the course of 4-6 hours for each feeding. Following acclimation, pairs of fish were transferred to experimental tanks (Figure 2) for behavioral trials.

Experimental Apparatus

The experimental apparatus was modeled after Boersma et al. (2008). Behavioral experiments took place in 0.5-m cubic flow-through glass tanks. Each tank was gravity-fed 8°C seawater at 2 $L \cdot min^{-1}$ and contained a 2-cm layer of sand. One experimental tank was used in the behavioral characterization experiment, while three identical tanks were used in the OA experiment. Opaque barriers were placed on the lateral sides of each tank to minimize the potential for fish disturbance and to facilitate controlled visual exposure to a predator during the behavioral characterization experiment. An opaque blind surrounding the experimental tank(s) minimized fish disturbance from external stimuli.

Air was bubbled into the tank through a 20.3-cm-long, 7-mm-diameter air stone attached to the tank. A length of flexible tubing was laterally attached to the air stone and

connected to a three-way port outside of the blind to facilitate olfactory cue introductions. The air stone and cue-delivery tube were positioned 1 cm above and parallel to the sediment surface. Their close proximity to one another and to the sediment surface ensured rapid mixing of introduced cues and ensured sanddab exposure to these cues at the sediment-water interface.

Food delivery occurred at two stations 21 cm apart along the midline of each tank. Air propelled each pellet through a 7-mm-diameter flexible tube and into a vertical 43-cm-long transparent tube. Pellets fell through the water column and out the end of the tube, becoming available for consumption for 3-5 s before falling through a mesh grate slightly elevated above the sediment surface. The grate prevented food from accumulating on the tank bottom, forcing fish to intercept food pellets from the water column. This experimental design enabled a clear identification of feeding activity, facilitating a more precise evaluation of fish foraging under different conditions.

A video camera was placed 0.6 m away from each experimental tank at ~ 45° angle to the sediment surface to record activity and evaluate fish posture relative to the sediment. An opaque barrier directly behind the camera(s) minimized disturbances to test fish. Observations and delivery of food and predation cues occurred from behind the barrier.

Potential Predation Cues

Potential predation cues included two olfactory cues and one visual cue, all of which have been shown to elicit antipredation responses in other fishes. Olfactory cues were seawater infused with either 1) predator odor (PO) from Pacific cod (*Gadus*

macrocephalus), a known predator of juvenile flatfishes, or with 2) damaged skin extract (DS) from injured conspecifics.

The PO cue was created by holding two sub-adult Pacific cod in 5 L of chilled seawater for 2-4 hrs; 120 ml of predator-conditioned seawater was used as the cue in the experiment. Two speckled sanddab were used to produce a dose of damaged skin extract (DS). Fish were euthanized using MS-222 and rinsed in seawater to remove chemical traces. A series of epidermal cuts (~150 mm total) were then made to each side of both fish. After 10 minutes of soaking in a beaker containing 100 ml of seawater, scored fish were removed and the cue-conditioned seawater was filtered through 350-µm mesh to remove scales. Cues were made individually for each trial of the behavioral characterization experiment, placed on ice, and used within 30 minutes of preparation. To minimize variation in cue characteristics between trials in the OA experiment, cues were prepared in batches rather than in single doses. Pairs of fish were sacrificed, rinsed, scored, and soaked as above. Water from multiple pairs was filtered, pooled, divided into 80-ml doses, and frozen at -20 °C until experimental use.

To standardize visual presentation of a predator (V), a weighted rubber flounder (34.3 cm x 16.5 cm) was used and maneuvered remotely around an identical water-filled tank adjacent to the experimental tank. An opaque barrier between the two tanks was raised and lowered remotely to control visual exposure to the model predator (Boersma et al. 2008).

Experimental Procedures

Behavioral Characterization Experiment

Sanddab were not permitted to eat 48 hrs prior to testing and were acclimated to experimental tanks overnight before trials began the following morning. Fish were always tested in pairs to facilitate normal behavior (as per Boersma et al. 2008). To ensure fish were active and responsive to stimuli, fish were presented with food multiple times for periods of up to 7 min during a pre-trial testing stage. Up to three 4-mm food pellets were presented to fish at 1-min intervals at alternating feeding stations for each food presentation (similar to Boersma et al. 2008). Trials commenced 1 hr after one of the two fish first attempted to capture a pellet.

Four cue types were used in this experiment, the three potential predation cues and a "blank" seawater control (SW). Pairs of fish were exposed to each of the four cues in 1-hr intervals in a randomized order. Behavioral observation was continuous within each "cue presentation" and was divided into four contiguous observation "periods": 1) pre-cue, 2) cue, 3) feeding, and 4) post-feeding. The "pre-cue" period consisted of 10 min of observation. For olfactory cues and the SW blank, the "cue" period consisted of 2 min, during which the cue was introduced over the first minute while the second minute allowed the cue to mix throughout the tank and acted as a buffer between cue and food introductions. The cue period during V cue presentation consisted of 5 min; 30 s to raise the barrier and expose fish to the sight of the model predator, 3 min of model predator manipulation, 30 s to replace the barrier, and 1 min to act as a buffer between cue and food introductions. The cue period was immediately followed by a 5-min "feeding"

period, during which single food pellets were introduced to alternating feeding tubes at 1-min intervals. This was immediately followed by another 10 min of observation in the "post-feeding" period (Figure 3). Fish lengths were recorded for each fish at the end of each trial and averaged 80.5 ± 1.2 mm standard length. Fish were not re-used in behavioral observations (n = 17).

Similar to Boersma et al. (2008), behavioral responses to cues were evaluated using video analysis by recording posture, activity level, feeding latency, and number of feeding strikes. Posture scores ranged from zero (flat and buried in the sediment) to five (positioned in the water column) and were recorded for each fish at thirty-second intervals. Average posture scores of each pair of fish were calculated for each period of each cue presentation. Activity level was assessed by placing a 3 x 3 grid over the video screen and counting the cumulative number of lines crossed by both fish in thirty-second intervals. Activity scores of each pair of fish were averaged over the thirty-second intervals of each observation period for each cue presentation. Differences in posture and activity levels were calculated between the "pre-cue" and subsequent periods (Δp and Δa) to account for each cue's effect on the behavioral responses of individual pairs of fish. Feeding latency, F_{min}, was quantified as the minute in which a fish first attempted to feed during the feeding period (score of 1-5). If neither fish attempted to feed, the pair was assigned a score of six. The number of feeding strikes on pellets during the feeding period (F_s) was recorded for each pair of fish regardless of the strike resulting in success.

The order in which fish experienced cues had no effect on their behavioral responses to the cues (all $p > 0.188$). Sanddab posture and activity during the pre-cue

periods did not differ among the four cue presentations (all $p > 0.4$). The effects of predation cues on both raw scores and changes in scores from pre-cue levels ("Δ-values") were determined using repeated-measures ANOVA with cue and trial treated as main effects and period as the repeated measure. Activity scores were log-transformed prior to analysis to meet statistical assumptions. Orthogonal linear comparisons to the behavior observed during the SW blank presentation were used to determine the effect of predation cues on sanddab posture and activity. Effects of cue on feeding latency and number of feeding strikes were analyzed using 1-way ANOVA. Average fish size and size-discrepancy between fish in the sanddab pair were analyzed as covariates on cue effects of behavior using ANCOVA.

Ocean Acidification Experiment

To determine the effects of elevated CO_2 levels on juvenile sanddab behavior, the experimental design from the previous experiment was modified to include 1) a CO_2 treatment variable and 2) the presentation of damaged skin cues from injured conspecifics (DS) to each pair of fish. Behavioral responses to DS cues were chosen to examine under elevated CO_2 conditions, because sanddab exhibited the most obviously consistent behaviors following exposure to this cue compared to the other cues during the previous experiment.

CO_2 levels were regulated by a pH meter (Honeywell Durafet II®) programmed to maintain desired pH. When the pH of the chilled seawater in the CO_2 conditioning tank increased past the set point, a solenoid dispersed ~4.5 cfm CO_2 into the conditioning tank for 0.4-0.8 s at 3-s intervals until desired pH was reached. A second conditioning tank

received chilled seawater at ambient CO_2 levels. Water from each conditioning tank was pumped to the header tanks in varying proportions to create three treatments with distinct CO_2 levels. The low CO_2 treatment contained seawater at ambient CO_2 levels. Medium and high CO_2 treatments generally corresponded with worst-case scenario CO_2 levels predicted to globally occur within the next 100 and 150 years, respectively (IPCC 2013; Table 1). CO_2 seawater treatments were gravity fed from header tanks to acclimation tanks at 1 $L \cdot min^{-1}$. Two water samples were collected from each treatment once a week throughout the experiment, poisoned with $MgCl_2$, and sent to the Ocean Acidification Research Center at the University of Alaska at Fairbanks. Total alkalinity (TA) and dissolved inorganic carbon (DIC) were quantified from samples using a VINDTA 3C (Versatile INstrument for the Determination of Total inorganic carbon and titration Alkalinity) and AIRICA (Automated InfraRed Inorganic Carbon Analyzer), respectively. pH, pCO_2, and aragonite saturation state (Ω) were calculated with the program, CO2CALC (Robbins et al. 2010).

Speckled sanddab were acclimated to experimental treatment CO_2 levels in two steps. Fish assigned to the high CO_2 treatment were first placed in tanks containing the medium CO_2 treatment, and on the following day were transferred to tanks containing the high CO_2 treatment. Fish assigned to the low and medium CO_2 treatments were placed directly in the tanks containing their assigned treatment; however, they were briefly captured and quickly returned to their acclimation tanks one day after the start of the acclimation period to standardize handling across treatments. During the CO_2 acclimation period, fish were also acclimated to the automated food delivery system as previously

described. All fish were fed in these tanks on the fourth day of acclimation and were fed at least ten times prior to experimentation over the course of 4-6 weeks. Fish were prevented from eating 48 hrs prior to testing and were acclimated overnight to experimental tanks containing seawater at their acclimated CO_2 level. Trials began the following morning. Fish were tested in pairs to foster normal behavior (Boersma et al. 2008).

Each trial contained three different testing stages occurring at one-hour intervals and are referred to as 1) baseline, 2) DS response, and 3) post-DS response stages. Each stage was comprised of the same four contiguous observation periods described above: 1) pre-cue, 2) cue, 3) feeding, and 4) post-feeding. Injection of a “blank” seawater (SW) was used during the first and third stages as a procedural control.

The baseline stage reflected sanddab behavioral responses to assigned CO_2 treatment. If sanddab did not attempt to feed during the baseline stage, they were retested up to five additional times over the course of two days (i.e. tested in one-hour intervals up to three times per day) until a feeding strike occurred. If fish did not attempt to feed by the sixth testing, the trial was ended.

To standardize feeding state of sanddab across trials, we evaluated the effect of CO_2 level in fish who had previously demonstrated a willingness to feed in the experimental system. Thus sanddab were not tested in the two subsequent stages unless they attempted to feed by striking at a food pellet. Behavioral responses exhibited during the testing and re-testing of the baseline stage during which a sanddab first attempted to feed, were characterized as “first-feeding” behaviors and were treated as a separate stage

during analyses. In cases where fish attempted to feed in the first baseline testing, the same data were used to represent both “baseline” and “first-feeding” behavior for that pair. Following the expression of first-feeding responses, sanddab were exposed to damaged skin cues to determine the effects of elevated CO_2 on the previously characterized behavioral responses to this cue. The post-DS response stage reflected the effects of elevated CO_2 on any lasting effects of DS exposure on sanddab behavior (Figure 4).

Average size of speckled sanddab was 90.3 ± 0.5 mm and did not vary significantly across CO_2 treatments. Fish were not reused in experiments (sample size of 36 sanddab pairs in each CO_2 treatment, except for 35 pairs in the medium CO_2 treatment). Several trials were excluded from analyses due to problems with video recordings. In one trial a fish made contact with the feeding tube and buried; this trial and a few similar trials were not included in analyses. Two additional trials were excluded from analyses due to fish jumping out of the tank during the experiment.

Posture, activity, latency to feeding, and number of feeding strikes were recorded and used to evaluate sanddab behavioral responses in the same manner as in the behavioral characterization experiment. An “attempt” at feeding metric, F_a, was also recorded for each pair of fish, and was quantified as the number of repeated tests of the “baseline” stage (score of 1-6) it took sanddab to exhibit first-feeding behavior. If neither fish attempted to feed by the sixth feeding “attempt,” the pair received a score of seven and were not subject to further testing.

The effects of CO_2 treatment on both raw scores and changes in scores ("*Δ*-values") were determined within each stage using repeated-measures ANOVA with CO_2 treated as the main effect and period as the repeated measure. Activity scores were log-transformed prior to analysis to meet statistical assumptions. Orthogonal linear comparisons to the behavior observed in fish from the low CO_2 treatment were used to determine the effect of elevated CO_2 on sanddab posture and activity. A survival analysis was used to determine the effect of CO_2 treatment on F_a. Effects of CO_2 treatment on feeding latency and feeding strikes were analyzed using 1-way ANOVA. Average fish size and size-discrepancy between fish in the sanddab pair were analyzed as covariates on CO_2 effects of behavior using ANCOVA.

Figures and Tables

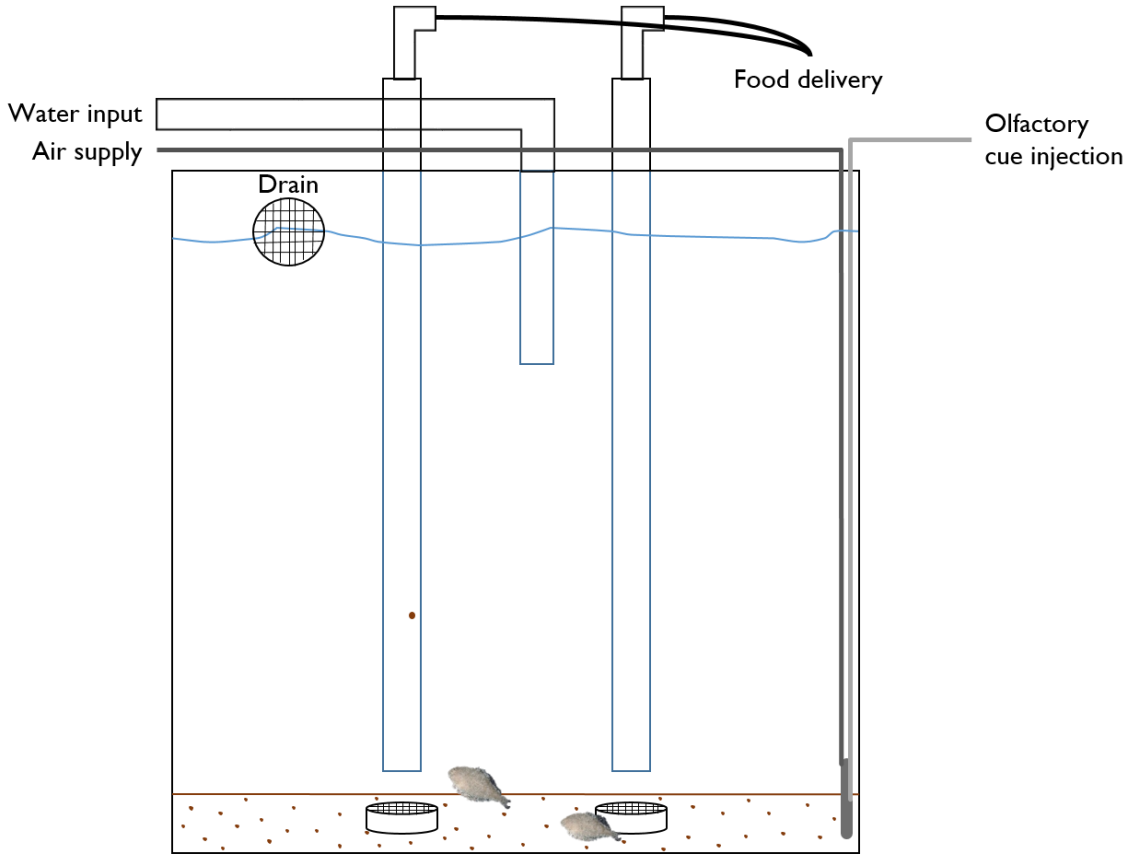

Figure 2. Schematic of the experimental apparatus. Experiments took place in 0.5-m cubic flow-through tanks. The food delivery system within the experimental tank is composed of two transparent tubes, each suspended several centimeters above a mesh grate. Flexible tubing delivered olfactory cues near the air stone to ensure adequate mixing of cues throughout the tank.

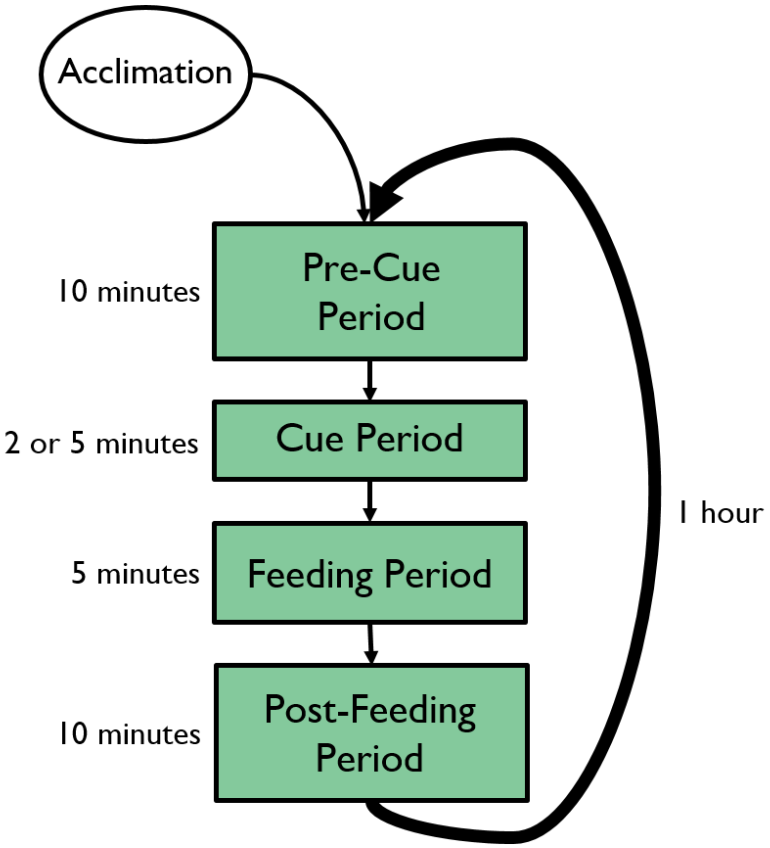

Figure 3. Schematic of a cue presentation. Cue presentations consisted of observation during four contiguous "periods" following acclimation to the experimental tank. Each trial consisted of four cue presentations corresponding to each of the different cue types. Cue presentations occurred at one-hour intervals with cue types presented in a randomized order.

Table 1. Conditions (mean ± standard error) during speckled sanddab acclimation to CO_2 treatments and during the ocean acidification experiment.

Metric	**Low (L) CO_2 Treatment**	**Medium (M) CO_2 Treatment**	**High (H) CO_2 Treatment**
Temperature (°C)	8.5 ± 0.1	8.5 ± 0.1	8.5 ± 0.1
DIC ($mol \cdot kg^{-1}$)	1960.8 ± 19.2	2061.1 ± 19.6	2115.3 ± 18.2
TA ($\mu mol \cdot kg^{-1}$)	2093.2 ± 20.5	2098.6 ± 20.7	2100.6 ± 18.8
pH (seawater scale)	8.01 ± 0.01	7.69 ± 0.02	7.50 ±.50 6
pCO_2 (µatm)	402 ± 14	910 ± 38	1445 ± 52
$\Omega_{Aragonite}$	1.55 ± 0.03	0.81 ± 0.05	0.53 ± 0.03

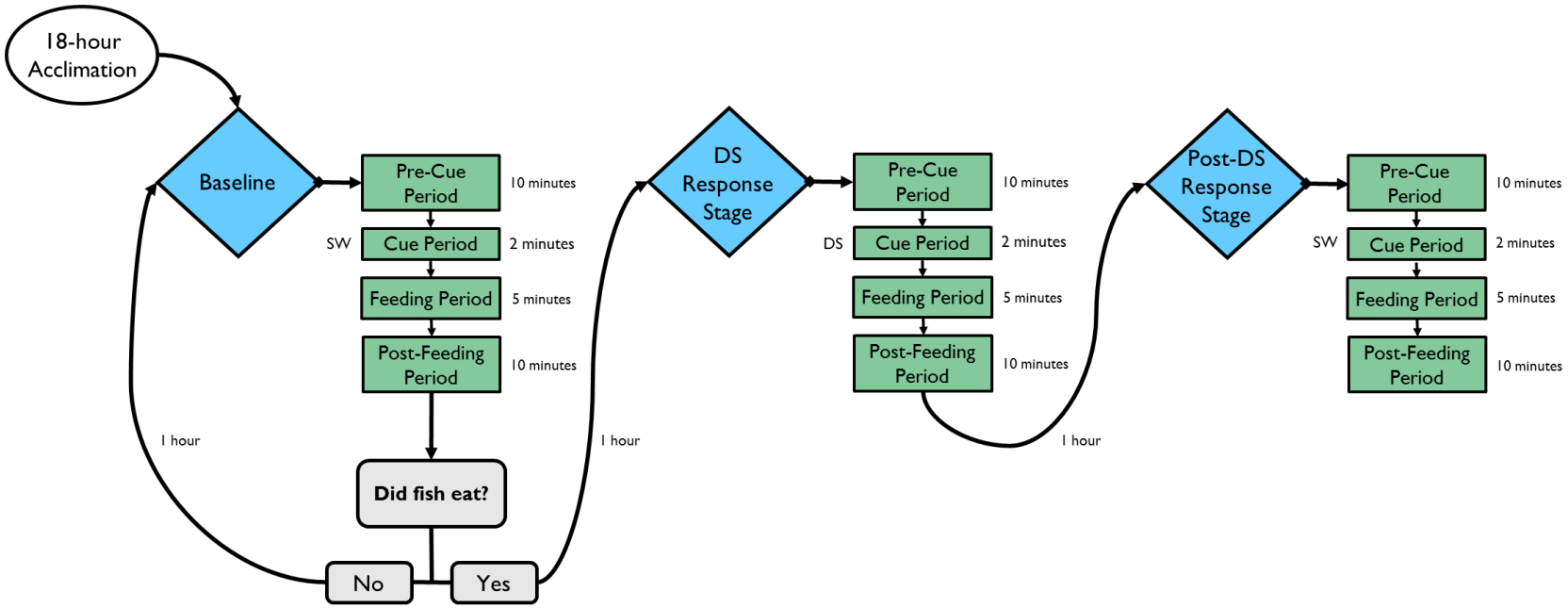

Figure 4. Schematic of a trial from the ocean acidification experiment. Sanddab first underwent an 18-hour acclimation to experimental tanks prior to experimentation. Trials consisted of up to three stages, each comprised of continuous observation during a cue presentation. Four contiguous periods comprised a cue presentation. A seawater blank (SW) and skin extract from damaged conspecifics (DS) were presented during the cue period of the corresponding stages. If a pair of fish did not attempt to feed during the (I) baseline stage, this stage was repeated at one-hour intervals. Once a fish attempted to feed, it was subsequently tested in the following two stages. The behavioral responses of a pair of fish during the repeated (if necessary) presentation of the (I) baseline stage when a fish first attempted to feed were characterized as their "first-feeding" behaviors. Although the behavior of some pairs of fish during the first stage were characterized as both their "baseline" and "first-feeding" behaviors, not all pairs of fish exhibited "first-feeding" behavior.

Chapter 3 – Results

Behavioral Characterization Experiment

Posture and Activity

Observations with "blank" SW controls characterized the basic behavior of speckled sanddab in the experimental conditions. Prior to introduction of food, fish posture and activity scores were generally low (averaging 1.131 and 0.182, respectively). Because there was high correlation between behavioral responses in the initial "pre-cue" period and those of subsequent periods reflecting variation in overall activity level among pairs of fish (all $r > 0.50$, $p < 0.05$), changes in posture and activity from the pre-cue period (Δp and Δa) were calculated for cue, feeding, and post-feeding periods to describe responses to each cue. Speckled sanddab posture and activity were unchanged following introduction of the "blank" SW control cue (*a priori* linear comparisons of SW Δp and Δa to 0, $p > 0.4$). The introduction of food elicited an increase in speckled sanddab posture and activity scores (both $p < 0.005$). Posture and activity slightly decreased in the ten minutes following food exposure (i.e. post-feeding period), but remained elevated above pre-cue levels (linear comparisons for both $p < 0.03$; RM ANOVA period effect for both Δp and Δa, $p < 0.001$; Table 2; Figure 5).

The introduction of potential predation cues had different effects on the behavioral patterns of speckled sanddab (Table 2; RM ANOVA cue-period interaction $p = 0.092$ for Δp, $p < 0.001$ for Δa). The increased posture and activity scores observed during the feeding and post-feeding periods of SW controls were not observed following

exposure to the V cue (*a priori* linear comparisons to SW, $p < 0.05$ except for post-feeding posture $p = 0.191$). In contrast, presentation of DS cues led to significantly increased activity scores during the cue and feeding periods compared to those observed following the SW control introduction (both $p < 0.003$). Similarly Δp during the feeding period was highest following DS exposure, although differences among cues were not significant (cue-period interaction $p = 0.092$). Exposure to the PO cue did not elicit changes in the behavior of speckled sanddab as posture and activity levels did not differ from those observed under SW controls (all $p > 0.15$).

Fish size had an effect on some aspects of behavior in the experiments. During the cue period, there was a negative effect of average size of a pair of fish on Δp (ANCOVA, $p = 0.006$; Figure 6a) and an interactive effect of fish size and cue on Δa ($p = 0.031$; Figure 7a). The interaction was primarily driven by DS cues, in which there was a positive effect of fish size on Δa that was absent from all other cue presentations. During the feeding period, a marginally significant interaction of size and cue on Δp ($p = 0.050$) was primarily driven by the visual cue in which there was a negative effect of fish size on Δp (Figure 6b). In contrast, the interactive effect of fish size and cue on Δa during the feeding period was primarily driven by the positive effect of fish size on this response during DS presentations ($p < 0.001$; Figure 7b).

Foraging

There was a significant effect of fish size on feeding behavior in the experiments; larger fish exhibited shorter feeding latencies ($p = 0.002$; Figure 8) and a greater number of feeding strikes ($p = 0.004$; Figure 9) than smaller fish. Speckled sanddab fed the most

following DS exposure; fish attempted to feed in 82% of DS trials compared to 63%, 59%, and 47% of PO, SW, and V trials, respectively. Furthermore, speckled sanddab tended to exhibit more feeding strikes following DS exposure than following exposure to any other cue ($p = 0.051$; Figure 9).

Ocean Acidification Experiment

Baseline and First-Feeding

There was little effect of CO_2 treatment level on speckled sanddab baseline behaviors (Table 3). Posture and activity during the pre-cue period were unaffected by CO_2 treatment (*a priori* linear comparisons to low CO_2 treatment, $p > 0.2$), averaging 0.238 and 0.019 among all fish, respectively. As observed in the behavioral characterization experiment, posture and activity remained low following introduction of the SW blank, but increased during food introduction. Activity then remained at elevated levels while sanddab posture increased slightly during the post-feeding period (RM ANOVA, both $p < 0.001$; Figure 10). However, there was no statistically significant difference in baseline behaviors among CO_2 treatments (both $p > 0.7$)

There was no effect of average size of a sanddab pair as a covariate, but size discrepancy within the pair affected behavior during the feeding period. As the difference in size between a fish pair increased, both Δp ($p = 0.022$) and Δa increased ($p = 0.051$), while feeding latency decreased ($p = 0.050$). Fish from the low CO_2 treatment tended to have shorter feeding latencies than fish from elevated CO_2 treatments (Figure 11), but this was not significant (survival analysis, $p = 0.5$). There was no effect of CO_2 treatment

or fish size on the number of feeding strikes ($p > 0.2$; Figure 12). However, there was a trend toward a larger percentage of trials resulting in fish attempting to feed from the medium CO_2 treatment than in the other CO_2 treatments; over half of the medium CO_2 treatment exposures (55%) resulted in fish attempting to feed compared to 44% and 36% of exposures in the low and high CO_2 treatments, respectively (Figure 13).

If a pair did not attempt to feed in the "baseline," the stage was repeated up to six times over two days. The majority of all fish attempted to feed during the first or second attempt (F_a = 1 or 2, 77%). CO_2 treatment had no effect on F_a (survival analysis, chi-square = 1.351, $p = 0.509$), but fish fed within the first two attempts in a higher percentage of trials from the low CO_2 treatment (85%) than from the medium (74%) or high (71%) treatments (Figure 14). There was no effect of fish size on F_a.

During the first-feeding stage, there was an effect of fish size and CO_2 treatment on feeding latency. Longer feeding latencies were observed in smaller fish ($p = 0.044$) and in fish from the medium CO_2 treatment ($p = 0.048$). There was no effect of CO_2 level or fish size on the number of feeding strikes (all $p > 0.07$; Figure 12). There was little effect of CO_2 on speckled sanddab first-feeding posture and activity. Similar to baseline behaviors, pre-cue period posture and activity were not affected by CO_2 treatment (all $p > 0.05$). Posture and activity were unaffected by SW control exposure, but increased during food introduction. While there was no statistical effect of CO_2 on behavior, sanddab from the medium CO_2 treatment tended to display the lowest posture scores throughout the first-feeding stage ($p > 0.1$; Figure 10). Fish size had no effect on first-feeding posture or activity.

Damaged Skin Response

Sanddab behavioral responses to the introduction of DS cues were unaffected by CO_2 treatment ($p > 0.7$). However, fish from the medium CO_2 treatment tended to exhibit the lowest posture and activity scores (all $p > 0.1$; Figure 10). As observed in the behavioral characterization experiment, DS cue introduction elicited increases in posture and activity, both of which increased further when food was subsequently introduced. Posture then decreased to levels similar to those of the cue period ($p < 0.001$). Activity also decreased, but remained above that of the cue and pre-cue periods ($p < 0.001$). There was no effect of CO_2 on foraging latency or number of strikes (Figure 12) following introduction of DS cues (both $p > 0.7$).

Fish size had an effect on sanddab posture during the pre-cue period and on Δa during the cue period. There was a positive trend of average fish size on posture during the pre-cue period (ANCOVA; $p = 0.060$). Pre-cue posture scores were affected by an interaction of CO_2 treatment and relative fish size in which a positive effect was seen in fish from the low CO_2 treatment ($p = 0.034$). An interactive effect of average fish size and CO_2 treatment on Δa levels ($p = 0.021$) occurred during the cue period and was primarily driven by a positive effect of fish size exhibited in the medium CO_2 treatment. However, difference in fish size had a negative effect on Δa during the cue period ($p = 0.019$). There was no effect of fish size on feeding latency or feeding strikes (all $p > 0.2$).

Post Damaged-Skin Response

There was no effect of CO_2 on lingering effects of DS cue exposure on speckled sanddab posture and activity (both $p > 0.2$). However, there was a non-significant trend

towards fish from the medium CO_2 treatment displaying the lowest posture scores (all $p > 0.2$; Figure 10). Similar to the behavior exhibited during the first-feeding stage, posture and activity were unaffected by SW control introduction, increased when food was present, and activity then decreased slightly during the post-feeding period ($p < 0.001$).

Although there was no effect of fish size on posture or activity, there was a marginal effect of the interaction of CO_2 and average fish size on feeding latency ($p = 0.050$). In the medium CO_2 treatment, larger fish exhibited shorter feeding latencies than smaller fish whereas in the low CO_2 treatment, larger fish exhibited longer feeding latencies. Average fish size had no effect on feeding latency in fish from the high CO_2 treatment. Relative fish size had no effect on feeding latency. There was no effect of CO_2 treatment, average fish size, or relative fish size on feeding strikes ($p > 0.15$; Figure 12).

Figures and Tables

Table 2. Primary statistics used to analyze results of the behavioral characterization experiment. Cue control was a seawater (SW) "blank." Potential predation cues included a visual cue (V), damaged skin cues from injured conspecifics (DS), and predator odor (PO). Each cue presentation was comprised of the pre-cue, cue, feeding, and post-feeding periods. Changes in posture and activity from pre-cue levels (Δp and Δa) during subsequent periods were used as metrics in analyses. Feeding latency (F_{min}) and feeding strikes (F_s) were measured during the feeding periods. RM ANOVA were used to determine the overall effect of cue on behaviors for each period. Orthogonal linear comparisons of behavioral responses to potential predation cues to those of the SW blank were used to determine directional difference of cues on behaviors. The "result" columns indicate a metric's estimate for a cue within a period relative to that of SW within the same period. The relationship of statistically significant (<, >) and non-significant trends (≤, ≥) of cue estimates were directionally depicted relative to that of SW for each period. ANCOVA were used to examine the effects of average standard length of a sanddab pair (*avgSL*) on behavioral responses within each period.

Overall effect of cue and period: RM ANOVA

			p-values		
Main Effects	RM	Stage	*cue*	*period*	*cue x period*
cue + trial	period	Δp	0.105	< 0.001	0.092
		Δa	< 0.001	< 0.001	< 0.001

Cue-specific results: Orthogonal linear comparisons to SW

		Cue Period		Feeding Period		Post-feeding Period	
Cue	Metric	*Result*	*p-value*	*Result*	*p-value*	*Result*	*p-value*
V	Δp		0.543	V < SW	0.047	V ≤ SW	0.191
	Δa	V ≤ SW	0.119	V < SW	0.013	V < SW	0.006
DS	Δp		0.310	DS ≥ SW	0.176		0.572
	Δa	DS > SW	< 0.001	DS > SW	0.003		0.365
V	Δp		0.311		0.467		0.485
	Δa	PO ≤ SW	0.173		0.661		0.236

Table 2 Continued.

Size effects on responses: ANCOVA

		p-values		
Period	Metric	*cue*	*avgSL*	*cue x avgSL*
cue	Δp	0.657	0.006	0.207
	Δa	< 0.001	0.796	0.031
feeding	Δp	0.008	0.427	0.050
	Δa	< 0.001	0.031	< 0.001
	F_{min}	0.170	0.002	0.924
	F_s	0.051	0.004	0.615
post-feeding	Δp	0.290	0.711	0.889
	Δa	0.002	0.278	0.099

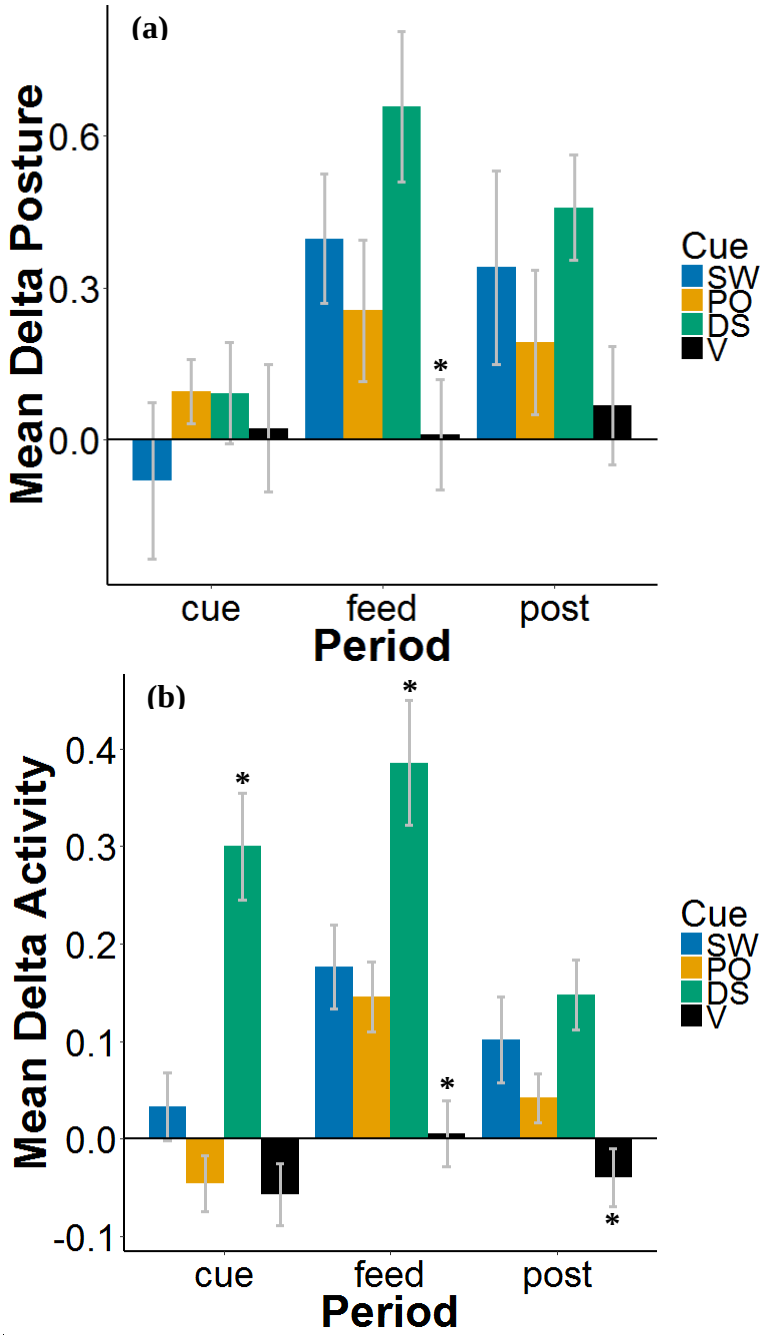

Figure 5. Changes in average speckled sanddab (a) posture and (b) activity (± standard error) from initial pre-cue levels in subsequent periods for each cue. Potential predation cues included predator odor (PO), damaged skin cues from injured conspecifics (DS), and the sight of a predator (V). A seawater (SW) blank was used as a cue control. Asterisks (*) indicate significantly different values from that of SW at a p-value < 0.05.

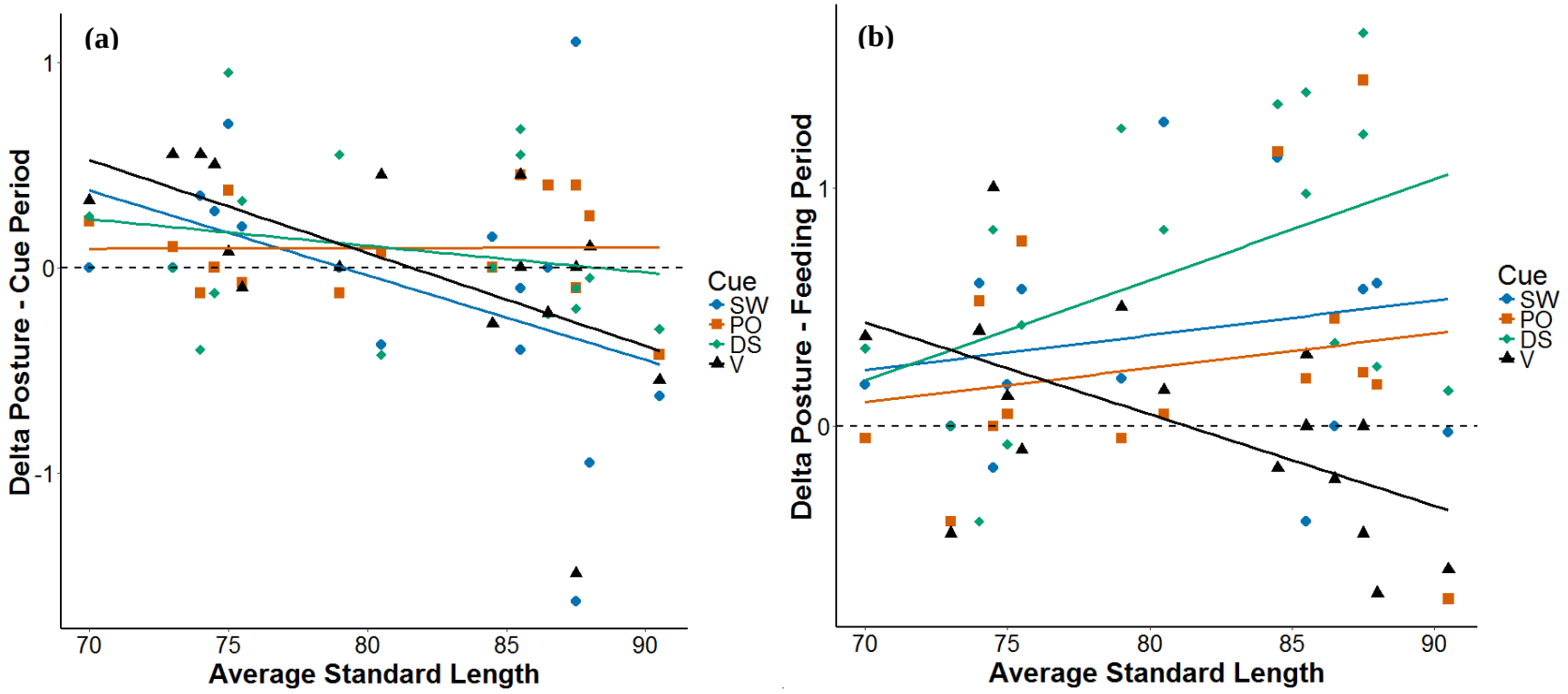

Figure 6. Changes in speckled sanddab posture from initial pre-cue period levels during the (a) cue and (b) feeding periods. Linear regressions between the average size of each pair of speckled sanddab and their behavioral responses are shown for each cue type of each trial. A seawater "blank" (SW) was used as a cue control. Potential predation cues included predator odor (PO), damaged skin cues (DS), and the sight of a predator (V).

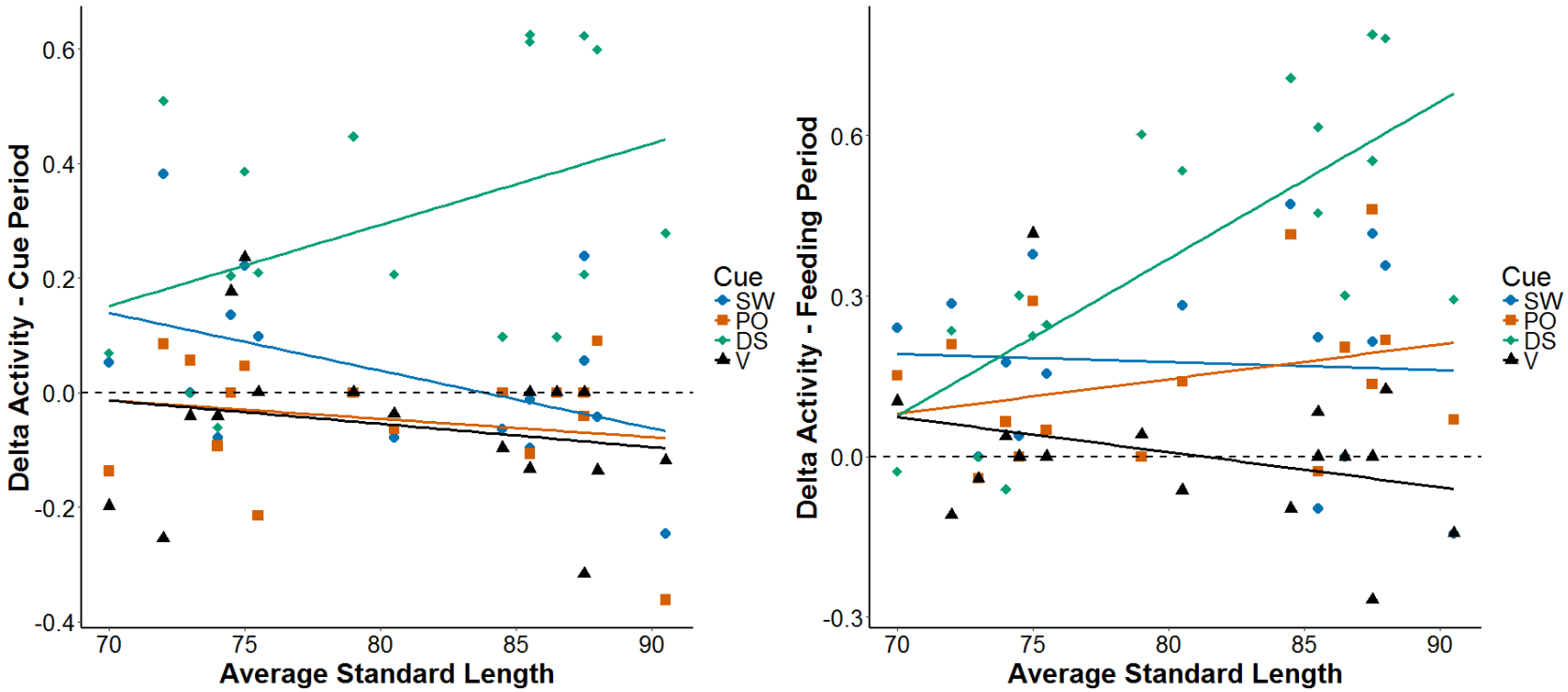

Figure 7. Changes in speckled sanddab activity from initial pre-cue period levels during the (a) cue and (b) feeding periods. Linear regressions between the average size of each pair of speckled sanddab and their behavioral responses are shown for each cue type of each trial. A seawater "blank" (SW) was used as a cue control. Potential predation cues included predator odor (PO), damaged skin cues (DS), and the sight of a predator (V).

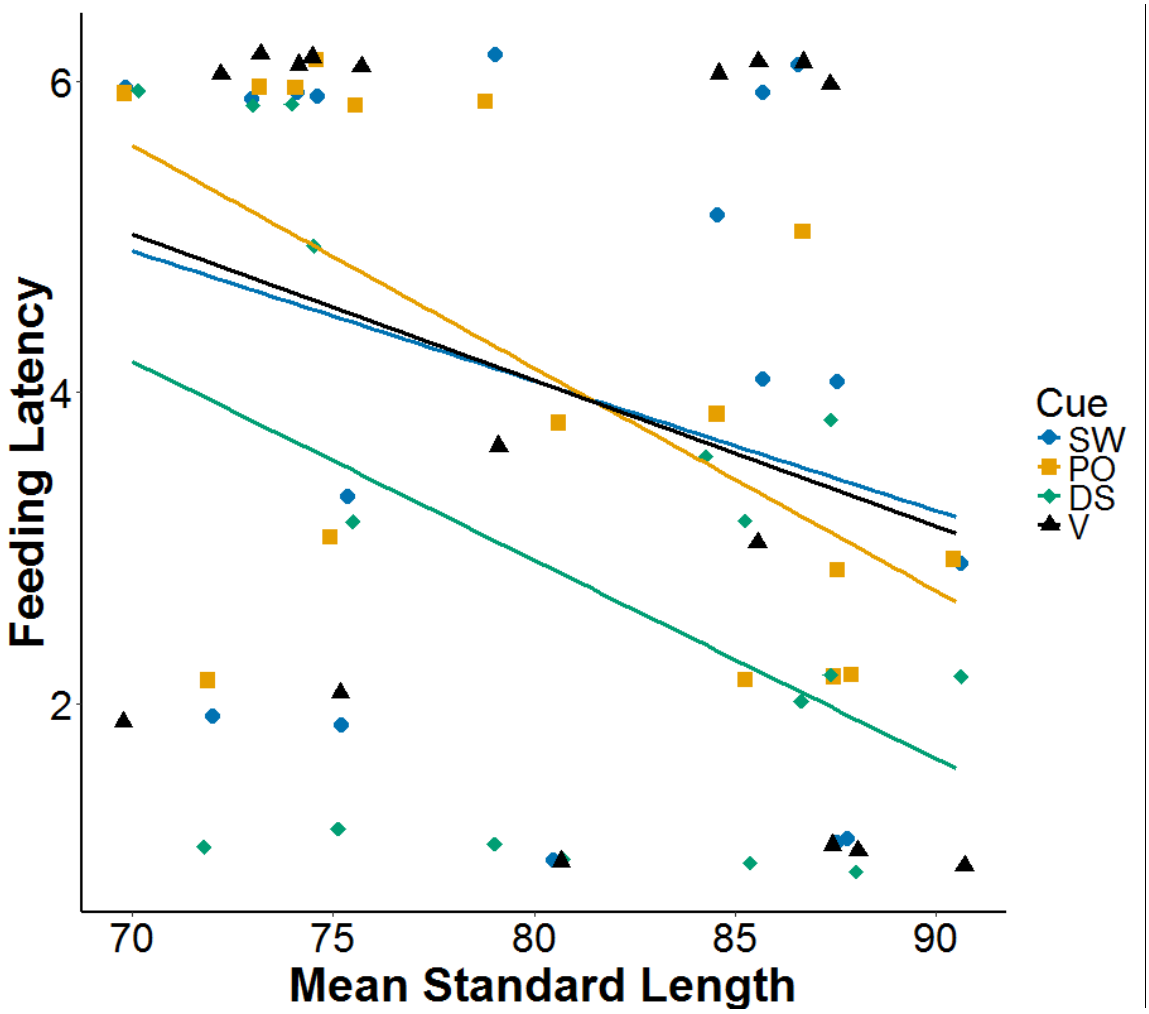

Figure 8. Linear regression of the average size of each pair of speckled sanddab with their feeding latency for each cue type. Feeding latency was quantified as the minute within the feeding period (score of 1-5) in which one of the two fish first attempted to feed. Fish that did not attempt to feed were assigned a score of 6. A seawater (SW) blank functioned as the cue control. Potential predation cues included predator odor (PO), damaged skin cues (DS), and the sight of a predator (V). Note: overlapping points are slightly offset for clarity.

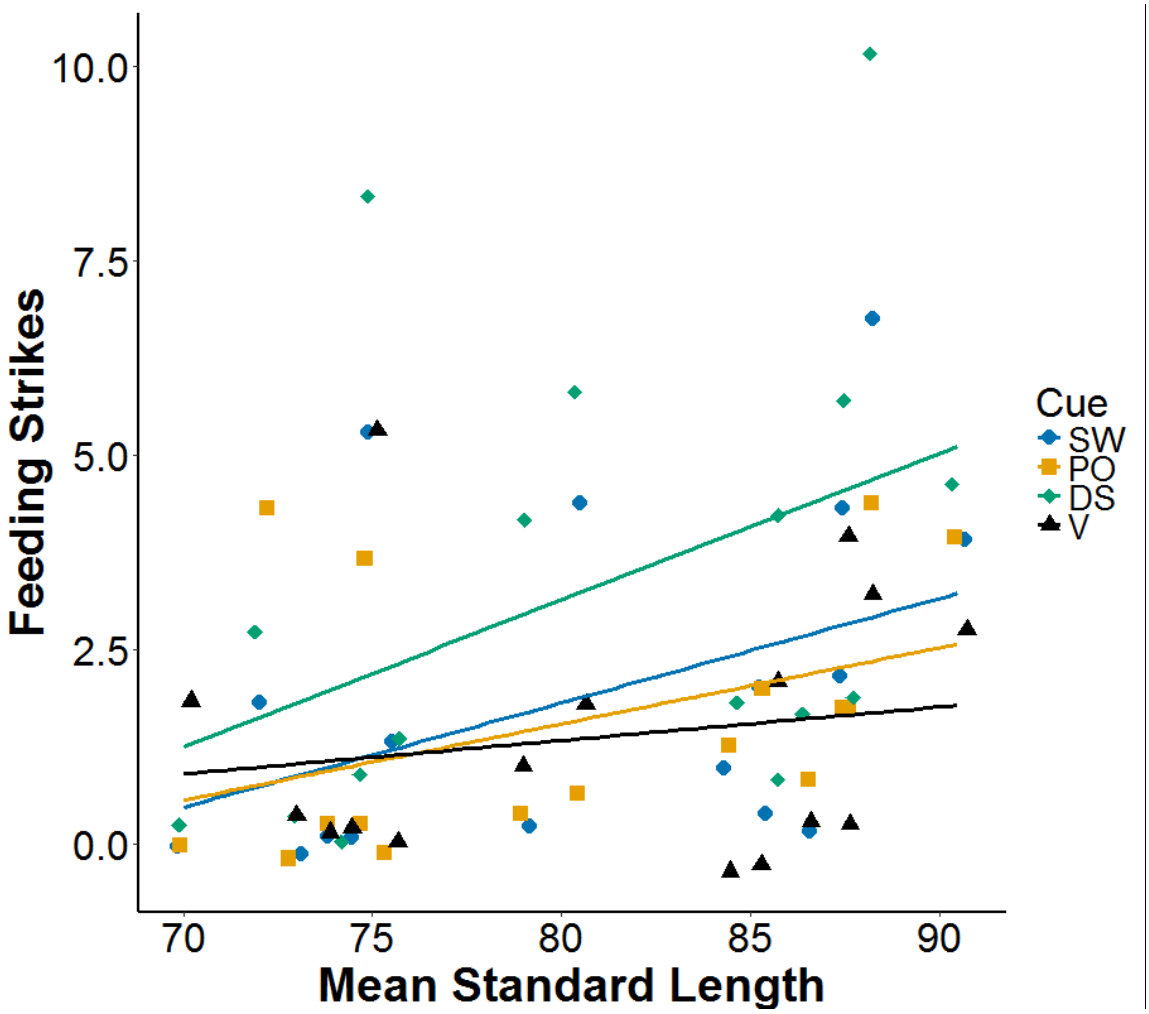

Figure 9. Linear regression of the average size of each pair of speckled sanddab with their total number of feeding strikes for each cue type. A seawater (SW) blank functioned as the cue control. Potential predation cues included predator odor (PO), damaged skin cues (DS), and the sight of a predator (V). Note: overlapping points are slightly offset for clarity.

Table 3. Primary statistics used to analyze results of the OA experiment. Trials consisted of baseline, first-feeding, DS response, and post-DS response stages. Each stage was comprised of pre-cue, cue, feeding, and post-feeding periods. Changes in posture and activity from pre-cue levels (Δp and Δa) during subsequent periods were used as metrics in analyses. Feeding latency (F_{min}) and feeding strikes (F_s) were measured during the feeding periods. Feeding attempt (F_a) scores of 1-6 indicate the baseline "attempt" in which a fish first attempted to feed with a score of 7 indicating that neither fish attempted to feed by the sixth attempt. RM ANOVA were used to determine the overall effect of CO_2 on behaviors for each stage. ANCOVA were used to examine the effects of average standard length of a sanddab pair (*avgSL*) on behavioral responses within each period of each stage. Survival analyses were used to determine the effect of CO_2 on F_a and F_{min}.

Overall effect of CO_2 during each stage: RM ANOVA

			Δp: p-values			Δa: p-values		
Main Effects	RM	Stage	*CO_2*	*period*	*CO_2 x period*	*CO_2*	*period*	*CO_2 x period*
CO_2	period	Baseline	0.882	< 0.001	0.444	0.746	< 0.001	0.943
		First-Feeding	0.437	< 0.001	0.609	0.59	< 0.001	0.886
		DS Response	0.631	< 0.001	0.722	0.564	< 0.001	0.762
		Post-DS Response	0.376	< 0.001	0.209	0.503	< 0.001	0.828

Table 3 Continued.

Size effects on responses: ANCOVA

		Cue Period p-values			Feeding Period p-values			Post-feeding Period p-values		
Stage	Metric	*CO_2*	*avgSL*	*CO_2 x avgSL*	*CO_2*	*avgSL*	*CO_2 x avgSL*	*CO_2*	*avgSL*	*CO_2 x avgSL*
Baseline	Δp	0.084	0.352	0.881	0.439	0.698	0.653	0.884	0.207	0.149
	Δa	0.426	0.472	0.845	0.751	0.597	0.513	0.865	0.391	0.504
	F_{min}				0.486	0.672	0.681			
	F_s				0.910	0.711	0.865			
First-Feeding	Δp	0.673	0.895	0.506	0.522	0.745	0.425	0.421	0.284	0.283
	Δa	0.429	0.177	0.706	0.651	0.947	0.604	0.730	0.469	0.863
	F_{min}				0.048	0.044	0.505			
	F_s				0.694	0.080	0.422			
DS Response	Δp	0.444	0.527	0.845	0.882	0.445	0.648	0.552	0.399	0.719
	Δa	0.467	0.229	0.021	0.672	0.717	0.122	0.728	0.185	0.435
	F_{min}				0.790	0.709	0.558			
	F_s				0.963	0.234	0.948			
Post-DS Response	Δp	0.842	0.495	0.972	0.270	0.587	0.233	0.271	0.523	0.742
	Δa	0.664	0.275	0.367	0.485	0.667	0.648	0.691	0.780	0.729
	F_{min}				0.608	0.850	0.050			
	F_s				0.349	0.163	0.663			

Table 3 Continued.

Effects of CO_2 on foraging responses: Survival analysis

Metric	Stage	p-values
F_a		0.509
F_{min}	Baseline	0.450
	First-Feeding	0.052
	DS Response	0.393
	Post-DS Response	0.292

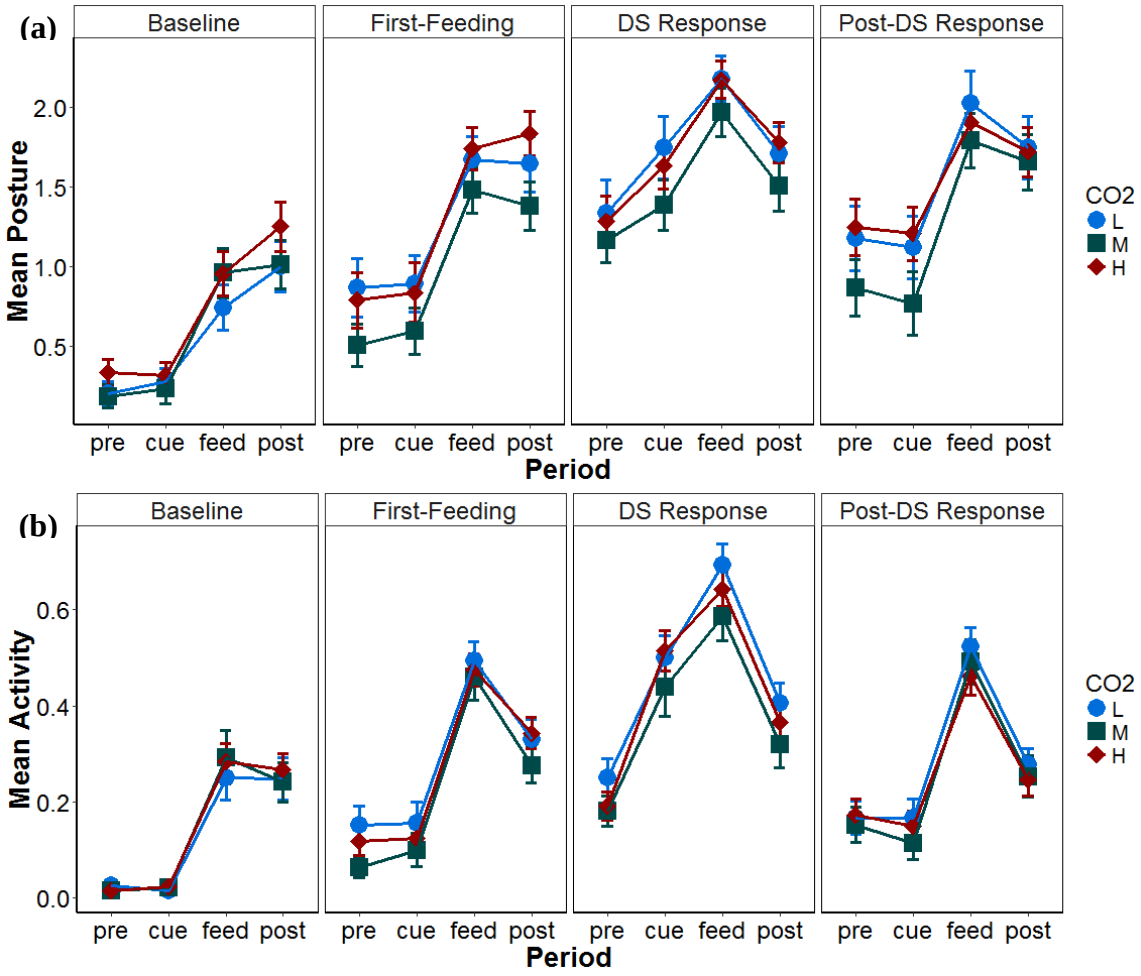

Figure 10. Average speckled sanddab (a) posture and (b) activity (± standard error) during each period of the Baseline, First-Feeding, DS Response, and Post-DS Response stages for each CO_2 treatment. Damaged skin (DS) cues were introduced during the cue period of the DS response stage. A seawater (SW) blank was introduced as a cue control during the cue periods of all other stages. The low (L) CO_2 treatment corresponded to present-day CO_2 levels. The medium (M) and high (H) CO_2 treatments corresponded to CO_2 levels predicted to occur over the next 100-150 years.

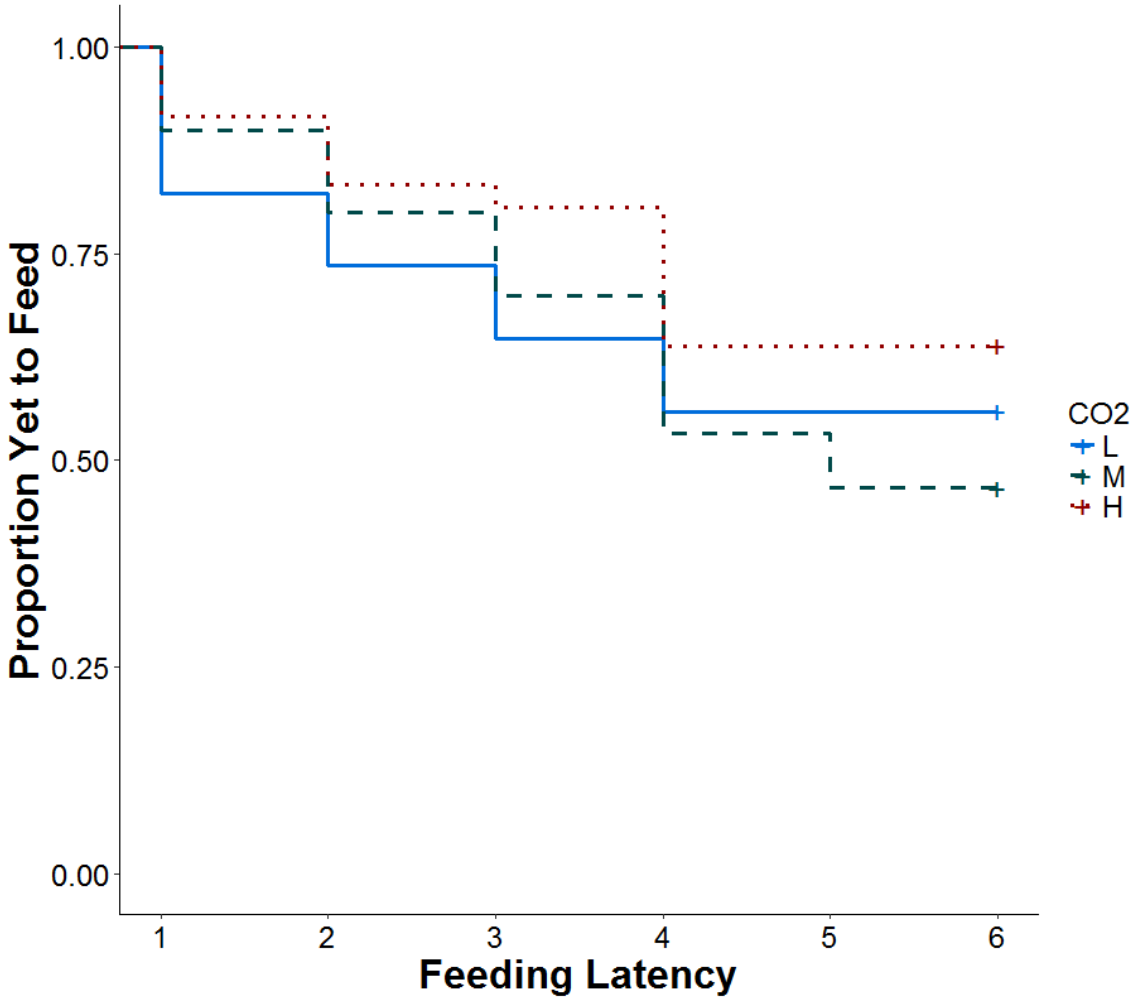

Figure 11. Proportion of fish yet to feed during each minute (score of 1-5) of the baseline feeding period. A feeding latency score of 6 indicates fish that did not attempt to feed by the end of the feeding period. The low (L) CO_2 treatment reflected present-day levels of CO_2 in seawater. The medium (M) and high (H) CO_2 treatments generally corresponded to levels predicted to occur within the next 100-150 years.

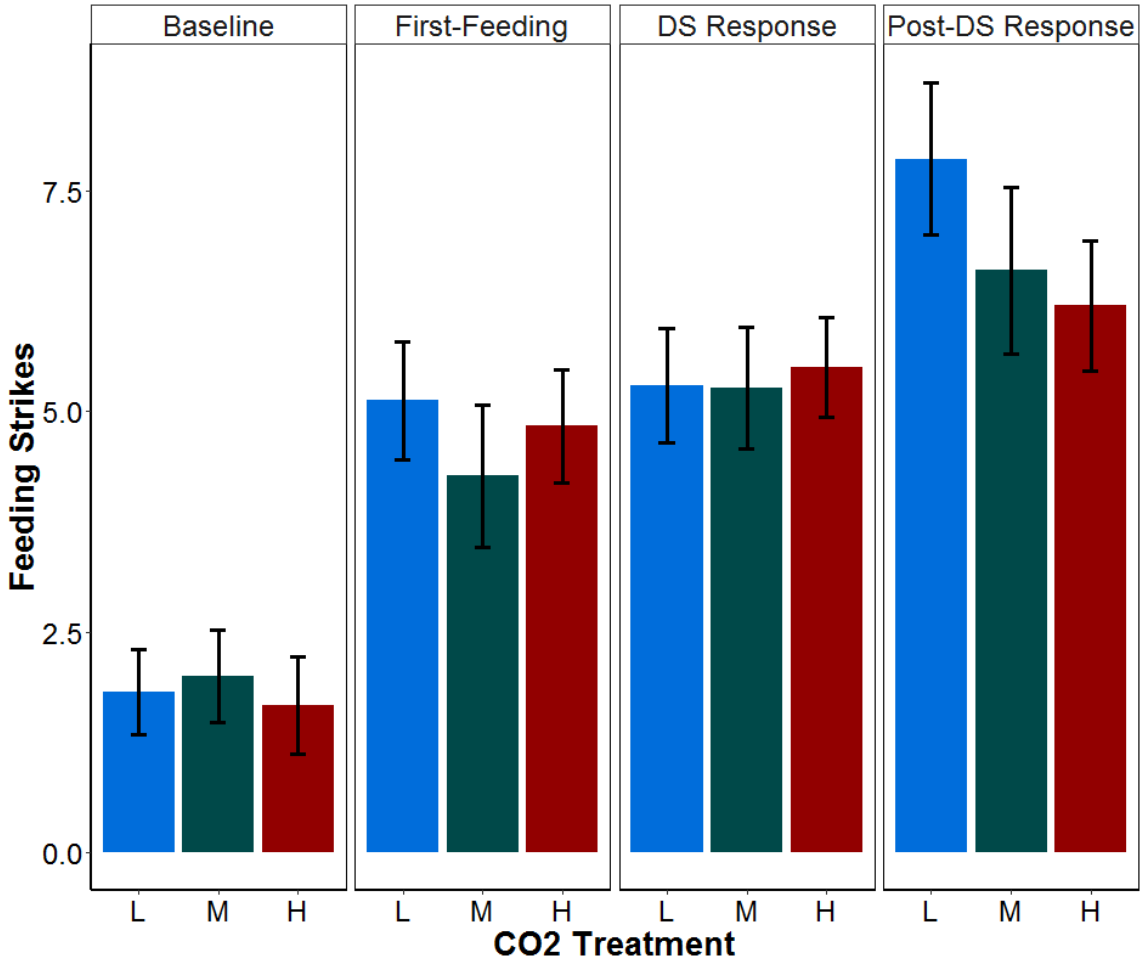

Figure 12. The average number of feeding strikes (± standard error) per CO_2 treatment within each stage. The low (L) CO_2 treatment reflected present-day CO_2 levels. Medium (M) and high (H) treatments generally corresponded to levels predicted to occur within the next 100-150 years.

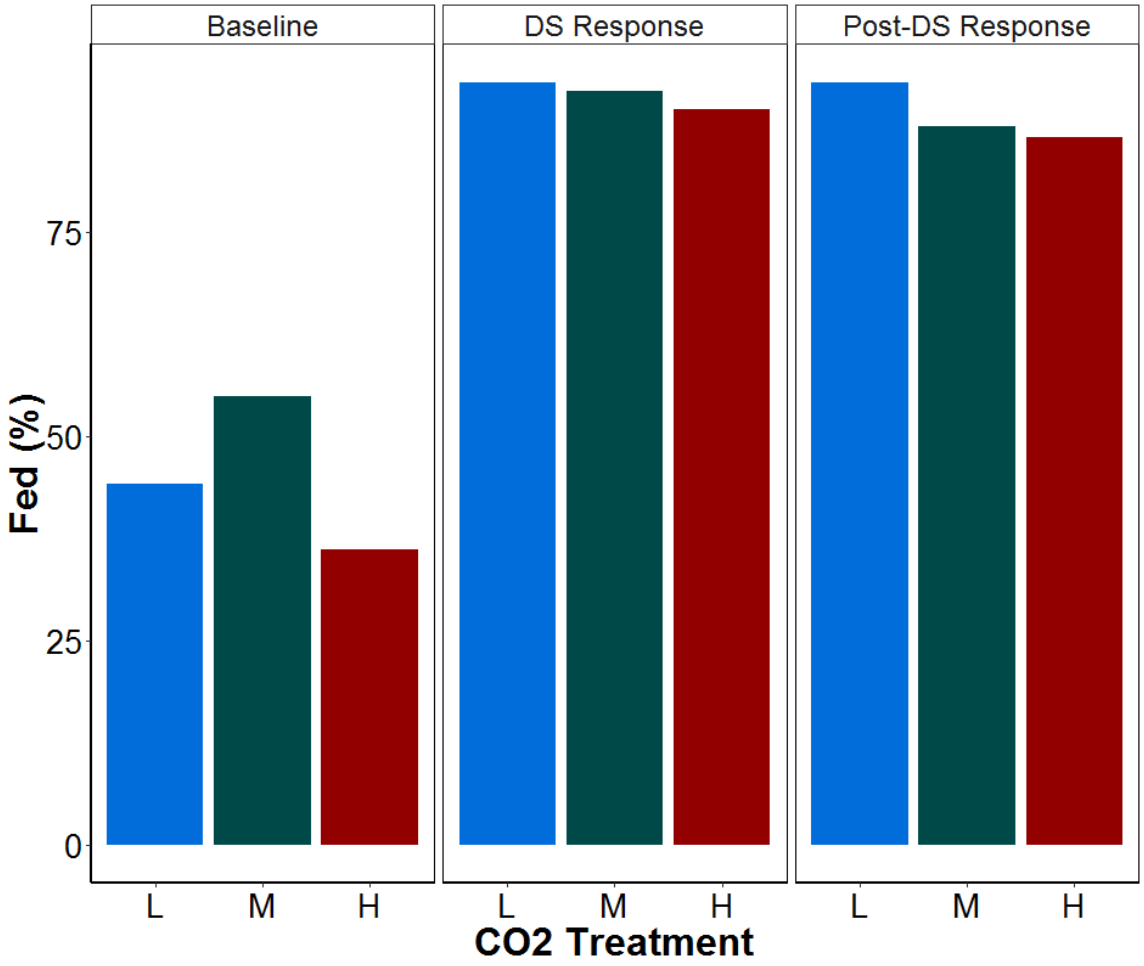

Figure 13. The percentage of trials resulting in a fish attempting to feed per CO_2 treatment within each stage. The low (L) CO_2 treatment reflected present-day CO_2 levels. Medium (M) and high (H) treatments generally corresponded to levels predicted to occur within the next 100-150 years.

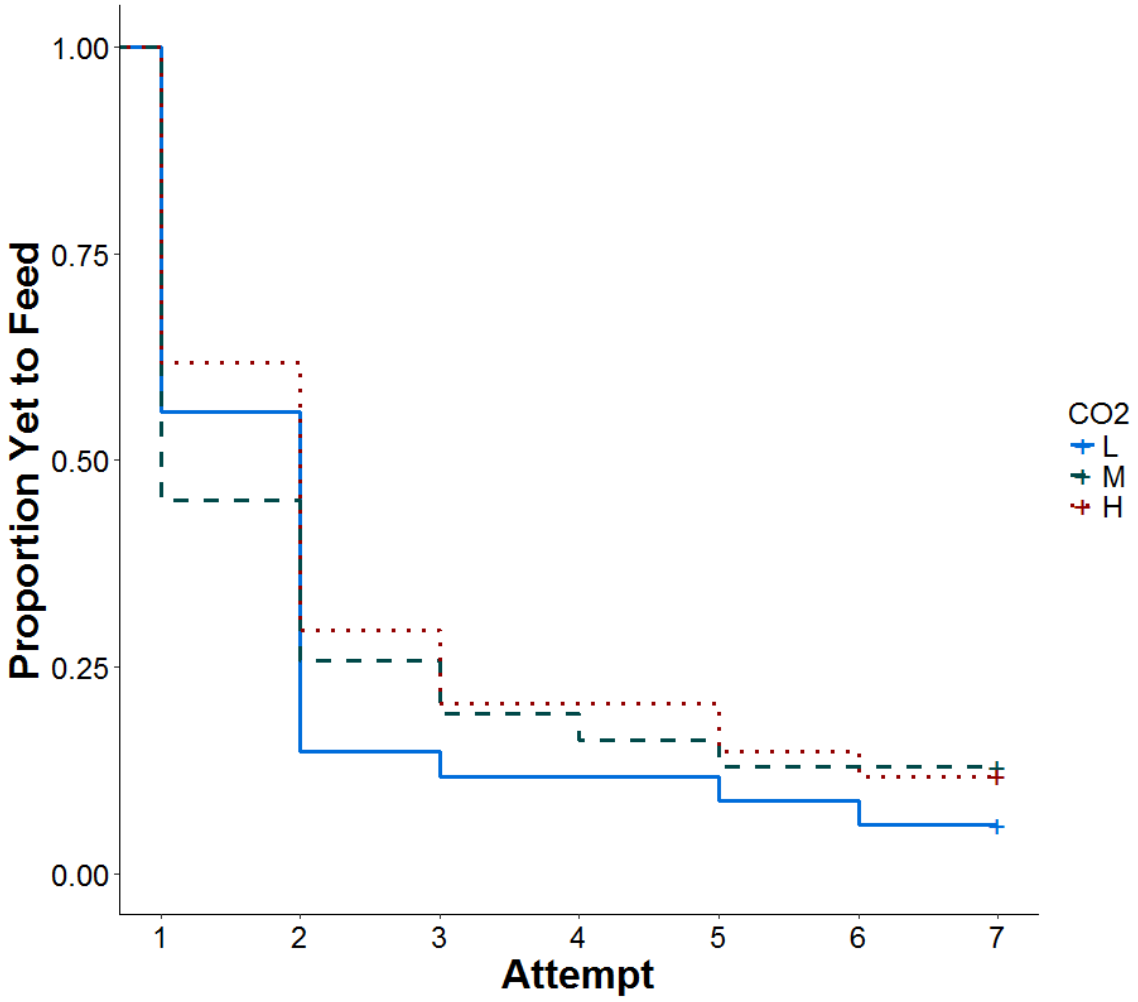

Figure 14. Proportion of fish yet to feed during each testing and retesting of the baseline attempt (score of 1-6). A feeding attempt score of 7 indicates fish that did not attempt to feed by the sixth testing (i.e. fifth retesting) of the baseline stage. The low (L) CO_2 treatment reflected present-day levels of CO_2 in seawater. The medium (M) and high (H) CO_2 treatments generally corresponded to levels predicted to occur within the next 100-150 years.

Chapter 4 – Discussion

To better understand the effects of ocean acidification on coastal fishes, I examined the responses of a flatfish to multiple potential predation cues. Speckled sanddab exhibited stereotypical anti-predator behaviors of reductions in conspicuousness following visual exposure to a predator. Unexpectedly, they exhibited elevated posture and active foraging behavior following exposure to damaged skin cues, and did not alter behavior in response to predator odor.

Elevated CO_2 levels appeared to have no major effect on sanddab behavior. However, fish from the elevated CO_2 treatments tended to have a decreased likelihood of exhibiting feeding within the first two feeding attempts compared with fish from the low CO_2 treatment. Furthermore, a non-significant trend towards the lowest posture and activity scores displayed in fish from the medium CO_2 treatment suggests that the effects of CO_2 on these behaviors may have been masked by effects on other related traits (e.g. growth). There are also the possibilities that sanddab are more resistant to elevated CO_2 than other fishes or that extended exposure to CO_2 treatments enabled speckled sanddab to fully acclimate and retain normal behaviors.

Behavioral Characterization

Speckled sanddab posture and activity were generally low in the absence of food and potential predation cues. As expected, food pellet introduction elicited increases in both posture and activity during the feeding period. Furthermore, larger fish tended to feed sooner and exhibit a greater number of feeding strikes than smaller fish. This was

expected as larger fish need to feed more than smaller fish to meet higher energetic demands and are less vulnerable to gape-limited predation.

As hypothesized, speckled sanddab reduced posture, activity, and foraging behaviors following exposure to the sight of a predator. These anti-predator behavioral responses were expected as they have been observed in other flatfishes (Boersma et al. 2008, Maia et al. 2008). Sanddab in some trials exhibited what appeared to be predator inspection behaviors not reported by Boersma et al. (2008). These events were characterized by an orientation and movement towards the model predator with elevated posture and were followed by several minutes of no movement. While these events might consist of sanddab exhibiting predator signaling, it seems unlikely as sanddab are both cryptic and not as strong of swimmers as other flatfishes such as Pacific halibut. I suggest that additional metrics (e.g. percentage of time spent in each section of the tank), would better characterize sanddab behavioral responses to the sight of a predator and could provide clarification on the nature of these events.

Speckled sanddab behavioral responses to PO exposure did not differ from those exhibited during blank SW injection. Pacific cod are known predators of juvenile flatfishes and thus it is unlikely that sanddab would not perceive them as a predation threat. It is difficult to determine and experimentally replicate the strength of olfactory cues that fish would experience in natural settings, but the extended holding of Pacific cod during PO production makes it likely that the PO cue was at least as strong as fish would experience in nature. It thus appears likely that sanddab would recognize Pacific cod odor as coming from a potential predator, but did not change their behavior when

exposed to this stimulus. The lack of a response may be associated with a cryptic lifestyle and living in turbid coastal and estuarine areas. These factors may confer perceived safety to speckled sanddab such that they will not suppress posture, activity, and feeding to some predation-associated stimuli (e.g. predator odor). Such an explanation was proposed for the lack of reduction in conspicuousness seen in English sole following exposure to the sight of a predator (Lemke and Ryer 2006a, Boersma et al. 2008). The results of subsequent lab experiments provided evidence supporting this hypothesis as English sole suffered lower predation mortality from sub-adult Pacific halibut in turbid waters compared with clear waters (Lemke and Ryer 2006b). There is also the possibility that Pacific cod odor is an unreliable indicator of predation threat to speckled sanddab as the scent of predators may be ubiquitous or at such low concentrations in nature that visual confirmation of a predator is necessary to elicit anti-predator behaviors.

Speckled sanddab exhibited an unexpected response to damaged skin extract from injured conspecifics. These cues are frequently referred to as “alarm cues” as they have been shown to elicit responses that are believed to minimize predation vulnerability in many species (Ferrari et al. 2010). However, sanddab exposure to the scent of injured conspecifics elicited increases in posture and activity, and thus they became more conspicuous. Furthermore, following exposure to DS cues, sanddab exhibited shorter feeding latencies and the same number of feeding strikes as when they were presented with food in the absence of potential predation cues (i.e. SW blank presentation). These results suggest that damaged skin from injured conspecifics may not function as a predation cue in juvenile speckled sanddab.

Studies of larval sturgeon (*Acipenser fulvescens*) also reported a significant increase in activity following DS exposure compared to activity in the absence of the cue (Wishengrad et al. 2014, Sloychuk et al. 2016), a result similar to that observed in speckled sanddab. The behavioral responses of larval sturgeon were interpreted by the authors as an escape response and as being different from responses to food cues. However, evidence supporting this interpretation is lacking as larval sturgeon foraging responses were not examined following exposure to damaged skin cues. While DS-induced increases in activity may plausibly be interpreted as an escape response for larval sturgeon, I suggest that this is not the case for juvenile speckled sanddab as they actively foraged following exposure to damaged skin cues. It is possible that DS cues function as a more general "alert" cue for juvenile speckled sanddab, causing them to locate the source of the cue prior to exhibiting appropriate behaviors. In this experiment, sanddab exposure to DS cues was always followed by the introduction of food and thus the majority of trials resulted in sanddab exhibiting behaviors associated with foraging. Perhaps if DS cue introduction were paired with or followed by exposure to the sight of a predator, speckled sanddab might have exhibited different behavioral responses to the cues in the presence and absence of food. There is also the possibility that DS cues are perceived as food cues by speckled sanddab. In laboratory experiments with juvenile Atlantic halibut (*Hippoglossus hippoglossus*), exposure to prey scent elicited orientation towards the odor and increased activity level as they swam around the tank for several minutes (Yacoob and Browman 2007). Because similar behavior was observed in

sanddab, it appears more likely that DS cues are perceived as food cues than as alert or predation cues by speckled sanddab.

Examining fish behavioral responses to the sight of a predator, Boersma et al. (2008) classified three species of flatfish as risk-averse (northern rock sole), risk-prone (English sole), and risk-sensitive (Pacific halibut). Assuming the sight of a predator was the only cue unambiguously perceived by sanddab as predation threat, they exhibited behaviors most similar to Pacific halibut. Both sanddab and halibut exhibited risk-sensitive behaviors characterized by foraging and elevated posture and activity in the absence of the predation threat, while significantly reducing these behaviors during and following exposure to the sight of a predator (Lemke and Ryer 2006a, Boersma et al. 2008).

Regardless of how speckled sanddab perceive predator odor, damaged skin cues, and the sight of a predator, these stimuli would be associated with a nearby, successful predation event, and are thus indicative of some level of predation risk to sanddab. Similar to English sole, speckled sanddab may have evolved to generally exhibit risk-prone behaviors in the presence of the aforementioned cues, likely due to habitat-mediated protection from turbid coastal waters (Lemke and Ryer 2006a, Lemke and Ryer 2006b, Boersma et al. 2008). Regardless of whether or not some speckled sanddab were exhibiting inspection or predator signaling behaviors when exposed to the sight of a predator, instances of elevated posture and movement toward the predator can be characterized as being risky as they would make the fish more conspicuous. The lack of suppressed behavioral responses to predator odor and the dramatic increase in

conspicuousness to damaged skin cues are probably further ways in which speckled sanddab have evolved to balance trade-offs between foraging and predation. Living in a turbid environment may offer cryptic sanddab additional protection from visual predation, enabling sanddab to actively forage and maintain growth.

Ocean Acidification Effects

In this study, the behavioral metrics examined in speckled sanddab were not significantly affected by CO_2 treatment and were similar to those exhibited during blank SW introduction of the behavioral characterization experiment. Many studies have demonstrated altered routine activity and foraging behaviors in fishes following exposure to elevated CO_2 (Munday et al. 2010, Cripps et al. 2011, Ferrari et al. 2011). In the only other study of OA behavioral effects in a marine flatfish, juvenile Senegalese sole (*Solea senegalensis*) exhibited altered lateralization and habitat preference (Sampaio et al. 2016). However, recent studies have demonstrated a lack of OA-induced effects in the behavior of some other fishes (Jutfelt and Hedgärde 2015, Heinrich et al. 2016, Sundin and Jutfelt 2016). Thus the lack of a major CO_2 effect on sanddab posture, activity, and foraging behaviors in the absence of potential predation cues suggests that certain aspects of speckled sanddab behavior may be resilient to future OA conditions.

Many studies of the effects of OA have focused on the responsiveness of marine fishes to olfactory cues. The majority of published studies have reported altered behavioral responses to olfactory foraging and predation cues following exposure to elevated CO_2 levels (Dixson et al. 2010, Munday et al. 2010, Dixson et al. 2014, Sundin

and Jutfelt 2016). Although a few studies of fishes have demonstrated no effect of elevated CO_2 on behavioral responses to olfactory cues (Jutfelt and Hedgärde 2013), OA studies are just starting to examine these effects in fishes from habitats other than coral reefs and could yet reveal similar responses.

Although there were no major effects of CO_2 on routine behaviors or behavioral responses to damaged skin cues from injured conspecifics, fish from the medium CO_2 treatment tended to exhibit lower posture and activity scores, longer feeding latencies, and later feeding attempts compared to fish from other CO_2 treatments. If sanddab are able to detect elevated CO_2 (Jutfelt and Hedgärde 2013) but are unable to avoid it, a reduction in movement and foraging could be a means of reducing energetic costs associated with maintaining internal pH when experiencing OA conditions. However, this reduction in behaviors was not seen in fish from the high CO_2 treatment as would be expected if this mechanism were occurring.

It has been hypothesized that elevated CO_2 alters behavioral responses to olfactory cues by impairing GABA-A neurotransmitter function (Nilsson et al. 2012). An alternative proposed mechanism behind these changes in behavior are due to pH altering the chemical structures of sensory-mediated molecules and associated receptor sites (Roggatz et al. 2016). While I did not test for the possibility of the latter proposed mechanism, I did not see evidence supporting this hypothesis as a trend in altered behavior was seen in fish from the medium CO_2 treatment rather than the high CO_2 treatment. Furthermore, trends in altered behavior were observed throughout the entire experiment, not solely during DS cue presentation. While I can not rule out compensation

of related responses masking the effects of elevated CO_2 on speckled sanddab behavior, the responses needed for this to ensue are not the most parsimonious explanation.

Identifying the phylogenetic or life history traits that influence species-specific sensitivity to elevated CO_2 will improve our understanding of which species might be more resilient as the ocean continues to accumulate CO_2. Speckled sanddab might be one of the species more resistant to elevated levels of CO_2 as their behavioral responses were generally resilient to elevated CO_2. Parts of the Oregon coast already experience elevated CO_2 levels that can increase when deep waters with naturally elevated CO_2 upwell into shallow, nearshore habitats (Feely et al. 2016). Potential transgenerational effects, acclimation, epigenetic effects, or a combination of these factors could confer a resistance to elevated CO_2. There is also the possibility that the length of acclimation to CO_2 treatments had an effect on the apparent sensitivity of sanddab behaviors to elevated CO_2. The majority of OA studies that have demonstrated dramatic effects of elevated CO_2 on fish behavior have had relatively short acclimation periods of a few days up to about a week, whereas several longer-term exposure studies have demonstrated less pronounced or no effects (Dupont and Pörtner 2013). These results may suggest that at least some of the changes in behaviors might be due to the short-term effects of altered CO_2 exposure. If that is the case, the longer acclimation period in our experiment of 4-6 weeks may have allowed sanddab to elevated CO_2 conditions, such that normal behaviors had been restored.

The potential resistance of speckled sanddab foraging behaviors to elevated CO_2 suggests that not all fishes face theoretical declines in survival. Future OA studies

examining the behaviors of more fishes from temperate and high-latitude regions are needed to compare behavioral responses of fishes occupying different habitats and ecological niches to better understand the effects of global climate change on marine fishes.

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