Behavioral responses of a coastal flatfish to predation-associated cues and elevated CO_2

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ABSTRACT

The direct and indirect effects of ocean acidification (OA) on marine ecosystems are a growing concern. Studies with marine fishes suggest that elevated CO_2 may affect behavior by interfering with an important brain neurotransmitter. OA effects on fish behavior are comparatively understudied in temperate and boreal species. In laboratory experiments, we first characterized 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 levels (~400 µatm). Sanddab reduced conspicuousness and foraging at the sight of a predator, but increased activity and conspicuousness when exposed to damaged skin cues. We then examined the effects of elevated CO_2 levels (~900 µatm and ~1,500 µatm) on posture, activity, and foraging of sanddab, and the behavioral responses to damaged skin cues. Sanddab behavior appeared generally resilient to the effects of elevated CO_2 levels, but there were non-significant trends of fish from the medium CO_2 treatment exhibiting lower posture and activity scores, and reduced feeding activity. The resiliency of speckled sanddab to OA conditions may be related to their distribution in a coastal upwelling region with seasonally elevated CO_2 levels. Alternatively, prolonged acclimation to elevated CO_2 may have mitigated the effects observed in other fishes following shorter-term exposures. Additional studies of ecologically relevant behaviors across diverse species assemblages are necessary to evaluate the impact of ocean acidification on marine food webs.

1. Introduction

Ocean acidification (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 atmospheric carbon dioxide increases, it dissolves into the oceans, leading to a decrease in the pH of marine waters. 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-driven change in the chemistry of the oceans is occurring faster than previous natural cycles in oceanic and atmospheric CO_2 levels (Gattuso and Hansson 2011). Elevated CO_2 levels have been shown to induce a variety of effects on marine organisms (Kroeker et al. 2013; Wittmann and Pörtner 2013), raising concerns about the cumulative impacts on productivity of marine ecosystems.

Marine fishes are generally expected to be more resilient to the effects of OA than invertebrate species with calcified exoskeletons (Melzner et al. 2009). However, empirical evidence has demonstrated considerable variation in the effects of elevated CO_2 levels on different fish species, life stages, and biological traits. For example, the growth and survival of larger-bodied juvenile and adult fishes appear generally robust to projected CO_2 levels (Hurst et al. 2012; Perry et al. 2015). Elevated CO_2 exposure has been shown to reduce growth, development, and survival of larval stages of some fish species (Bauman et al. 2012; Frommel et al. 2011; Hurst et al. 2015), but not others (Munday et al. 2011; Hurst et al. 2013; Bignami et al. 2013). However, OA effects in fishes are not limited to larval growth and development.

A number of studies have demonstrated OA-induced alterations of behavioral responses to environmental stimuli (Clements and Hunt 2015; Ashur et al. 2017), which are thought to result from an OA-induced impairment of $GABA_A$ neuroreceptors (Nilsson et al. 2012; Tresguerres and Hamilton 2017). For example, exposure to elevated CO_2 has been shown to alter behavioral responses to olfactory foraging cues (i.e., prey scent; Cripps et al. 2011; Dixson et al. 2014) and predation cues (i.e., odor from a predator or damaged skin cues from injured conspecifics; Dixson et al. 2010; Munday et al. 2010; Ferrari et al. 2011; Lönnstedt et al. 2013). Failure to recognize and respond to these foraging and predation cues can reduce growth rates and increase predation vulnerability (Munday et al. 2010).

Some studies have examined the effects of OA on behaviors with unknown or ambiguous ecological relevance such as behavioral lateralization. Changes in the degree of behavioral lateralization have been observed in fishes following high CO_2 exposure (Domenici et al. 2012; Nilsson et al. 2012). However, hypothesized advantages of behavioral lateralization are highly contextually dependent (Vallortigara and Rogers 2005), thus the ecological implications of altered lateralization are unclear. Similarly, “novel object tests” are typically interpreted as a measure of “boldness” (Carter et al. 2013), but the relevance of such responses could vary markedly across species with different life histories inhabiting structurally complex or uniform habitats. Testing known, ecologically relevant behaviors under elevated CO_2 levels is crucial to understanding the potential for OA to impact species interactions and population dynamics in marine ecosystems.

The majority of reports documenting high CO_2-induced behavioral disruptions have come from tropical reef species (Clements and Hunt 2015). Similar effects have been seen in some (Jutfelt et al. 2013; Sundin and Jutfelt 2016) but not all (Jutfelt and Hedgärde 2013; Sundin and Jutfelt 2016; Kwan et al. 2017) studies of temperate species, although the effects of OA on fish behavior remain comparatively understudied in fishes from temperate and high-latitude regions.

OA studies on fishes from these regions are especially important as they support many of the world's largest commercial fisheries and some, such as the California Current, are experiencing more rapid than anticipated progression of OA (Hauri et al. 2013; Feely et al. 2016). Flatfishes are an important component of these ecosystems, supporting regionally valuable fisheries and serving as central links within food webs. Experiments have revealed variation in physiological effects of elevated CO_2 on early life stages of flatfish species (Chambers et al. 2014; Gräns et al. 2014; Hurst et al. 2015); however, only one study to date has examined the effects of OA on aspects of flatfish behavior (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 body orientation. These traits minimize the profiles of flatfish on the sediment thereby reducing conspicuousness and predator detection. Because the act of foraging involves movement that could attract the attention of a predator (e.g. elevated posture and activity), foraging activity represents a direct contrast to anti-predation behaviors. Laboratory experiments have demonstrated that while several species of North Pacific flatfishes rely on reduced activity and burial in the sediment to avoid predation, there is variation among species within this general anti-predation strategy (Boersma et al. 2008; Lemke and Ryer 2006a, b). These experiments primarily relied on visual exposure to a predator; no study to date has examined flatfish behavioral responses to olfactory cues of predation risk.

We examined the behavioral patterns and effects of elevated CO_2 in the flatfish species speckled sanddab (*Citharichthys stigmaeus*). The study consisted of two independent laboratory experiments examining speckled sanddab behavior and foraging activity. We first characterized speckled sanddab behavioral responses to potential predation cues under ambient CO_2 levels. We then examined the effects of elevated CO_2 levels on aspects of routine behavior and foraging and the behavioral response to damaged skin cues (Ferrari et al. 2010), a widely applied approach in studies of OA effects on fishes. Results of these studies demonstrate that the effects of high CO_2 on marine fishes are likely to be species- and trait-specific and may include non-linear responses to CO_2 concentration. Further, the counter-intuitive response of speckled sanddab to the damaged skin cues demonstrates that interpretation of the potential impacts of OA-induced behavioral disturbances requires careful consideration and improved understanding of basic behavioral ecology of subject species.

2. Methods

2.1 Collection and Maintenance

Age-0 speckled sanddab were collected in Yaquina Bay, Oregon, at depths of 2-15 m using a 2-m otter trawl with a 3-mm mesh codend towed at 1-2 knots. Fish were held at the Hatfield Marine Science Center in flow-through tanks at 7-9°C and 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. During initial holding, fish were fed a gelatinized mixture of krill, squid, fish, and nutritional supplements to satiation three times per week.

Prior to use in behavioral experiments, groups of four fish were transferred to 100-L acclimation tanks. These tanks were used to acclimate fish to treatment CO_2 levels (CO_2 experiment, see below) and the remote food delivery method similar to that used in experimental trials. Three times per week, food was delivered throughout the day using belt feeders attached to a transparent vertical tube extending to the middle of the water column. Fish were able to see the food particle as soon as it entered the tube but could not capture it until it fell from the end of the tube. In addition to replicating the delivery system used in behavioral trials, this method delivered food at unpredictable times and removed any association of food introduction with external stimuli (e.g., experimenter presence). During acclimation and experiments, pelletized food (Bio-Oregon BioDiet®) was used to standardize feeding rates and particle presentation. Belt feeders delivered a combination of 2-mm and 3-mm pellets to each tank over the course of 4-6 hours for each feeding. Fish were acclimated to the feeding system for at least two weeks prior to use in behavioral trials.

2.2 Experimental Apparatus

The experimental apparatus was modeled after that of Boersma et al. (2008). Experimental tanks were glass 0.5-m cubes, each filled to a volume of 115 L. 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. Observations and delivery of food and potential predation cues occurred from behind the barrier.

Food delivery occurred at two stations 21 cm apart along the midline of the tank (Fig. 1). 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 behavior, facilitating a more precise evaluation of sanddab foraging. A video camera was placed 0.6 m away from each experimental tank angled at ~45° to the sediment surface to record activity and evaluate fish posture relative to the sediment.

2.3 Potential Predation Cues

Potential predation cues included 1) predator odor (PO) from Pacific cod (*Gadus macrocephalus*), a known predator of juvenile flatfishes throughout the North Pacific, 2) damaged skin (DS) cues from injured conspecifics, and 3) visual exposure to a predator (V). These cue types have been shown to elicit anti-predation responses in a range of other fishes (e.g., McCormick and Manassa 2008; Ferrari et al. 2010).

The PO cue was created by holding two sub-adult Pacific cod (30-40 cm) in 10 L of static, chilled seawater for 2-4 h; 120 ml of predator-conditioned seawater was used as the cue in the experiment. Two speckled sanddab of similar size to experimental fish were used to produce a dose of damaged skin extract (DS). Fish were euthanized using tricaine methanesulfonate (MS-222) and rinsed in seawater to remove chemical traces. A series of superficial cuts to the epidermis (~150 mm total) were then made to each side of both fish. After 10 min 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. 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. Fish were sacrificed, rinsed, scored, and soaked as above. Water from multiple fish was pooled, filtered, divided into 80-ml doses, and frozen at -20 °C until experimental use.

The olfactory predation cues were introduced to the tank through flexible tubing from behind the blind. The olfactory delivery tube was laterally attached to the air stone and connected to a three-way port outside of the blind. The air stone and cue-delivery tube were positioned 1 cm above the sediment surface to ensure rapid mixing of the cue as validated by testing the dispersal of dye.

To standardize visual presentation of a predator (V), a weighted rubber flounder (34.3 cm) was 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).

2.4 Experimental Procedures

2.4.1 Behavioral Characterization Experiment

Sanddab were fasted for 48 h prior to testing and were acclimated to experimental tanks overnight before behavioral observations (~16 h). 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 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 h 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-h 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 of routine behavior. For olfactory cues and the SW control, the “cue” period was 2 min; the cue was introduced during the first minute while the second minute allowed the cue to mix throughout the tank. The cue period during the V-cue presentation was 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. The feeding period was immediately followed by another 10 min of routine behavior observation in the "post-feeding" period (Fig. 3). Fish lengths, recorded for each fish at the end of each of the 17 trials (34 fish), averaged 80.5 ± 7.1 SE mm standard length. Fish were not re-used in behavioral trials.

Video recordings of trials were analyzed for behavioral responses to cues, including posture, activity, feeding latency, and number of feeding strikes (Boersma et al. 2008). Posture scores ranged from 0 (flat and buried in the sediment) to 5 (positioned in the water column) and were recorded for each fish at 30-s intervals. Activity level was assessed by counting the cumulative number of line-crosses of a 3-by-3 grid in 30-s intervals. Activity scores of each pair of fish were log-transformed to meet statistical assumptions. Average posture and log-transformed activity level of each fish pair was calculated for each observation period of each cue presentation. To isolate the effect of the cue on the behavior of individual pairs of fish, we calculated the change in posture and log-transformed activity levels between the "pre-cue" and subsequent periods (Δp and Δa). 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 latency score of 6. The number of feeding strikes on pellets during the feeding period (F_s) was recorded for each pair of fish regardless of whether the strike resulted in consumption.

The effects of potential predation cues on behavior (*p* and *a*) and changes in behavior (Δp and Δa) were determined using repeated-measures ANOVA with cue and trial treated as main effects and period as the repeated measure. Orthogonal linear comparisons to the behavior observed following SW control were used to determine the effect of potential predation cues on sanddab behavior. Effects of the cues 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.

2.4.2 Ocean Acidification Experiment

To determine the effects of elevated CO_2 levels on juvenile sanddab behavior, the previously described experimental design was modified to focus on response to the damaged skin (DS) cues, incorporating three CO_2 levels (one control and two elevated) as experimental treatments. Elevated CO_2 levels were maintained by automated injection of CO_2 into a conditioning tank based on continuous measurement of pH (Honeywell Durafet II®). A second conditioning tank received chilled seawater at ambient CO_2 levels. Water from each conditioning tank was pumped to three header tanks in varying proportions to create three treatments with distinct CO_2 levels. The low (L) CO_2 treatment contained seawater with ambient CO_2 levels (~400 µatm) and served as an experimental control while medium (M) and high (H) treatments were maintained at levels similar to those predicted to occur near the end of the century (900 µatm and 1,500 µatm; Table 1; IPCC 2013). Two water samples were collected from each treatment once per week throughout the experiment, treated 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 using a VINDTA 3C (Versatile INstrument for the Determination of Total inorganic carbon and titration Alkalinity) and AIRICA (Automated

InfraRed Inorganic Carbon Analyzer). The pH (seawater scale), 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 and automated feeding protocol for 4-6 weeks prior to behavioral trials. All fish were fed in these tanks at least ten times prior to experimentation. Fish were fasted for 48 h prior to testing and were acclimated overnight to experimental tanks containing seawater at their designated CO_2 level. Trials began the following morning. Fish were tested in pairs as described above.

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

The baseline stage reflected the effect of CO_2 treatment on overall activity level and willingness to feed. 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. one-hour intervals up to three times per day) until a feeding strike occurred. The number of repeated tests of the baseline stage conducted prior to the first feeding attempt by the fish (F_a) was recorded for each trial (score of 1-6). If neither fish attempted to feed by the sixth repeated test of the baseline stage, the pair received a score of 7 and was not subject to further testing. The responses exhibited during the test 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 DS cues to determine the effects of elevated CO_2 on the previously characterized behavioral responses to this cue. The post-DS response stage reflected whether elevated CO_2 affected longer duration effects of DS exposure on sanddab behavior (Fig. 4). We conducted 36 trials at each CO_2 level (total $n = 216$ fish or 108 pairs of fish). Data from all or part of several trials was excluded from analyses due to video recording problems or disruptions in the experimental procedures (each CO_2 treatment final $n \geq 28$ for all variables). Average size of speckled sanddab used in the OA experiment was 90.3 ± 7.7 SE mm and did not vary significantly across CO_2 treatments. Fish were not reused in experimental trials.

Sanddab posture, activity, feeding latency, and number of feeding strikes were recorded throughout each trial as described for the behavioral characterization experiment. The effects of CO_2 treatment on behavior were determined within each stage using repeated-measures ANOVA with CO_2 treatment as the main effect and period as the repeated measure and orthogonal linear comparisons of the behavior observed in the low CO_2 treatment to that observed in elevated CO_2 treatments. 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.

3. Results

3.1 Behavioral Characterization Experiment

3.1.1 Posture

Because patterns of posture and activity were highly correlated ($r = 0.765$, $p < 0.001$), we focused the analyses here on posture metrics as a reflection of flatfish behavior (consistent with Boersma et al. 2008). Observations with SW controls characterized the basic behavior of speckled sanddab in the experimental conditions. Prior to food introduction, posture scores were generally low, averaging 1.1 across all trials, and posture was unaffected by the introduction of the SW cue control (*a priori* linear comparisons of SW Δp to 0, p = 0.653). The introduction of food elicited an increase in speckled sanddab posture (p = 0.003; Fig. 4). Posture decreased slightly during the post-feeding period, but remained elevated above pre-cue levels (linear comparisons, p = 0.017; RM ANOVA, period effect p = 0.092; Table 2).

The introduction of potential predation cues had distinct effects on the behavior of speckled sanddab (Table 2; RM ANOVA cue-period interaction p = 0.092). Exposure to the visual predation cue suppressed the behavioral response to food availability, i.e. there was no increase in posture during the feeding stage (Fig. 4; *a priori* linear comparisons, p = 0.047). Similarly, posture scores during the post-feeding period were lower (but not significantly different) following the V cue than the SW control (p = 0.191).

Speckled sanddab responded in a counter-intuitive manner to the damaged skin cue. Following DS exposure, Δp during the feeding period was 60.3% higher than following the SW control, although this difference was not significant (p = 0.176).

Exposure to the PO cue did not affect sanddab behavior; behavioral responses were similar to those observed under SW controls (all comparisons of PO to SW, p > 0.15).

Fish size had an effect on some aspects of behavior (Table 2). During the cue period, there was a negative effect of size on Δp (p = 0.006; Fig. 5). During the feeding period, a marginally significant interaction of size and cue on Δp (p = 0.050) was primarily driven by a negative effect of fish size on Δp, but only following V cue presentation (Fig. 5).

3.1.2 Foraging

There was a significant effect of fish size on feeding behavior in the experiments; larger fish exhibited shorter feeding latencies ($p = 0.002$; Fig. 6) and a greater number of feeding strikes ($p = 0.004$; Fig. 7) 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 ($p = 0.051$; Fig. 7).

3.2 Ocean Acidification Experiment

3.2.1 Baseline and First-Feeding

There was little effect of CO_2 treatment on speckled sanddab baseline posture (RM ANOVA, CO_2–period interaction $p = 0.40$; CO_2 main effect $p = 0.88$; Table 3; Fig. 8). Pre-cue posture was generally low, averaging 0.2 across treatments. As observed in the behavioral characterization experiment, posture remained low following introduction of the SW blank, but increased during food introduction. Posture then remained elevated during the post-feeding period (RM ANOVA, period effect $p < 0.001$; Fig. 8). There was no effect of mean size of the sanddab pair on posture scores (ANCOVA, all $p > 0.2$), but size discrepancy within the pair affected behavior during the feeding period. Trials with a larger difference in size between fish in the pair had higher Δp scores ($p = 0.022$) and shorter feeding latencies ($p = 0.050$).

There was no effect of CO_2 treatment or fish size on the number of feeding strikes in the baseline stage ($p > 0.7$; Fig. 10). However, there was a nonsignificant trend toward fish from the low CO_2 treatment exhibiting shorter feeding latencies than fish from the two elevated CO_2 treatments (Fig. 9; survival analysis, $p = 0.45$). The majority of trials from all CO_2 treatments (77%) were characterized by fish feeding during the first or second attempt. 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 (Fig. 12).

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 treatment or fish size on number of feeding strikes (all $p > 0.05$). Similar to baseline behaviors, CO_2 had little effect on speckled sanddab first-feeding posture (RM ANOVA, $p = 0.4$). While there was no significant effect of CO_2 on posture, sanddab from the medium CO_2 treatment tended to display the lowest posture scores throughout the first-feeding stage (*a priori* linear comparisons to L, $p > 0.1$; Fig. 8). Fish size had no effect on first-feeding posture (all $p > 0.2$).

3.2.2 Damaged Skin Response

As observed in the behavioral characterization experiment, DS cue introduction elicited an increase in posture, which increased further when food was introduced. Sanddab behavior during the DS stage did not differ significantly among CO_2 treatments (RM ANOVA, $p = 0.631$). However, as in the first-feeding stage, fish from the medium CO_2 treatment tended to exhibit the lowest posture scores (Fig. 8). There was no effect of CO_2 on feeding latency ($p = 0.393$) or number of strikes following introduction of DS cues ($p = 0.790$).

Fish size had an effect on sanddab posture during the pre-cue period. A positive effect of relative fish size on pre-cue posture was seen in fish from the low CO_2 treatment (CO_2-dSL interaction, $p = 0.034$). There was no effect of fish size on feeding latency or number of feeding strikes (all $p > 0.2$).

3.2.3 Post-Damaged Skin Response

CO_2 level had no effect on sanddab posture in the post-DS stage (RM ANOVA, $p = 0.376$). However, again, there was a trend toward fish from the medium CO_2 treatment displaying the lowest posture scores. Although there was no effect of fish size on posture in the Post-DS stage, there was a marginally significant interaction between CO_2 and 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 (no effect of size in the high CO_2 treatment). There was no effect of CO_2 treatment, average fish size, or relative fish size on feeding strikes (all $p > 0.15$; Fig. 10).

4. Discussion

Experimental studies of the responses of marine fishes to potential feeding and predation cues have been widely used to examine the potential for OA-induced sensory and behavioral disturbances (Leduc et al. 2013; Clements and Hunt 2015). In particular, the reaction to scent of damaged skin from a conspecific has been widely applied. Here, we examined the effect of CO_2 on the feeding and behavioral response of a coastal flatfish to these damaged skin cues. As the behavioral response of juvenile flatfishes to olfactory predation cues has not previously been examined, we first characterized the response of speckled sanddab to multiple potential predation cues under ambient CO_2 conditions. Counter to our expectations, and observations made on other species, speckled sanddab exhibited elevated posture and actively foraged following exposure to damaged skin cues. We saw little effect of elevated CO_2 levels on sanddab behavior, suggesting species-specific variation in sensitivity or a potential for acclimation following extended exposure to elevated CO_2 levels. These experiments highlight the importance of understanding the context of fish behavioral responses in evaluating environmental effects on marine communities.

4.1 Behavioral Characterization

Speckled sanddab exhibited patterns of anti-predation behavior typical of those observed in other flatfishes and cryptic demersal fishes. Even in the absence of specific predation cues, activity levels were low and sanddab generally adopted a low posture on (or under) the sediment surface. Posture increased during foraging periods and fish made brief forays away from the sediment surface to capture prey items. As hypothesized, speckled sanddab reduced posture, activity, and foraging behaviors following visual exposure to a model predator. These anti-predation behavioral responses have been observed in other flatfishes (Boersma et al. 2008; Maia et al. 2008). Interestingly, some sanddab exhibited possible predator inspection behavior (Dugatkin et al. 2005). 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. This behavior was not reported by Boersma et al. (2008) in their study of other Pacific coast species. As this behavior increases conspicuousness and sanddab are cryptic without strong swimming capabilities (compared to species such as Pacific halibut), it is unlikely that juveniles would engage in active predator signaling. Therefore, predator inspection appears the most likely explanation, although additional work would be required for a more complete characterization of this aspect of behavior.

Speckled sanddab did not alter behavior (compared to SW controls) when exposed to predator odor. 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 and characteristics of olfactory cues that fish would experience in natural settings. Experimental research has shown that some prey species are more sensitive to olfactory cues when the predator has been fed the same prey species (Dixson et al. 2012). Pacific cod consume a diverse diet of fish and invertebrate prey. Although the cod in our experiment were not fed speckled sanddab we believe that in our experimental exposures the PO cue was generally similar to, and at least as strong as the olfactory predator cues that prey fish would experience in nature. Rather, living in turbid estuarine waters may result in a decreased behavioral sensitivity to some predation cues. High turbidity reduces the predation risk of juvenile flatfishes (Lemke and Ryer 2006a) which may result in a more "risk prone" suite of behaviors. Such an explanation was proposed for English sole (*Parophrys vetulus*) which did not increase anti-predation behavior even following visual exposure to a predator (Lemke and Ryer 2006a; Boersma et al. 2008). In contrast, the presence of predator odor may be insufficiently specific to indicate an immediate predation risk; 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-predation behaviors (Lima and Steury 2005).

Speckled sanddab exhibited an unexpected response to damaged skin cues from injured conspecifics. Damaged skin cues are frequently referred to as "alarm cues," as they have been shown to elicit responses that are believed to minimize predation vulnerability in a wide variety of marine and freshwater species (Ferrari et al. 2010). However, in sanddab, exposure to the scent of injured conspecifics elicited increases in posture and activity, which would presumably make fish more conspicuous and vulnerable to predation. Exposure to DS cues also resulted in shorter feeding latencies. These results suggest that damaged skin cues from injured conspecifics do 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 cue exposure (Wishengrad et al. 2014; Sloychuk et al. 2016). The behavioral responses of larval sturgeon were interpreted by the authors as an escape response and as being distinct from responses to food cues (although foraging responses were not characterized in the study). While DS cue-induced increases in activity may plausibly be interpreted as an escape response for larval sturgeon, we suggest that this is not the case for juvenile speckled sanddab as they actively foraged following exposure to damaged skin cues. Furthermore, studies have shown that behavioral responses to DS cues can be dependent on factors such as a fish's environment, life history stage, and hunger state (Harvey & Brown 2004; McCormick and Larson 2008; Chivers et al. 2014). It is possible that rather than triggering a general suppression of activity, DS cues function as a more general "alert" cue for juvenile speckled sanddab. If so, exposure to DS cue may have facilitated recognition and response to subsequent stimuli, which in these trials was food introduction. Finally, it is possible that DS cues are perceived by speckled sanddab as food cues.

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, *Lepidopsetta polyxystra*), risk-prone (English sole), and risk-sensitive (Pacific halibut). Although our testing procedures differed from those of Boersma et al. (2008), a similar visual exposure to the same model predator was used in both studies. We suggest that speckled sanddab 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 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).

4.2 Ocean Acidification Effects

Numerous recent studies have demonstrated altered behavior in marine fishes following exposure to elevated CO_2, potentially leading to widespread disruptions of marine ecosystems (Briffa et al. 2012). Many of these studies have focused on the responsiveness of marine fishes to olfactory cues (e.g. Munday et al. 2010; Cripps et al. 2011; Ferrari et al. 2011). However, other studies have documented little or no effect of elevated CO_2 level on responsiveness to olfactory cues or other aspects of behavior (e.g. Maneja et al. 2013; Heinrich et al. 2016; Sundin and Jutfelt 2016). Where they occur, these effects are hypothesized to be due to hypercapnia-induced disruption of $GABA_A$ neuroreceptor function (Nilsson et al. 2012; Tresguerres and Hamilton 2017).

Although we found no major effects of elevated CO_2 on routine behavior of speckled sanddab or their responses to damaged skin cues from injured conspecifics, fish from the medium CO_2 treatment tended to exhibit lower posture and activity scores compared to fish from other CO_2 treatments. Such a non-linear dose-response pattern could indicate a behavioral compensation that is only induced at a higher degree of CO_2 exposure. Fishes can detect (and respond to) CO_2 concentrations in seawater (Jutfelt and Hedgärde 2013). As a result, the cumulative effects of exposure to a wide range of CO_2 conditions could include both physiological and behavioral components resulting in non-linear organism level response patterns. In fact, one of the earliest studies of the effects of elevated CO_2 on fish behavior (Munday et al. 2009) found qualitatively different effects depending on the degree of acidification. Later studies have similarly found non-linear response patterns to elevated CO_2 on fish foraging behavior (Cripps et al. 2011) and growth (Silva et al. 2016). Unfortunately, because most studies of OA effects on fish behavior have incorporated only a single acidified treatment, it is difficult to know how common such non-linear responses may be. We recommend the incorporation of multiple treatments to better understand the CO_2 range over which behavioral disturbances are induced as well as consideration of potential compensatory responses.

Identifying the phylogenetic or life history traits that influence species-specific sensitivity to elevated CO_2 will improve our understanding of the cumulative impacts of ocean acidification to marine communities. In the only other study of OA effects on behavior in a marine flatfish, juvenile Senegalese sole (*Solea senegalensis*) exhibited altered lateralization and habitat preference following exposure to elevated CO_2 levels when combined with exposure to elevated temperature and/or methyl mercury contamination (Sampaio et al. 2016). The general resiliency observed in the behavioral traits of speckled sanddab to elevated CO_2 may be associated with their distribution in coastal waters of the U.S. West Coast. Although no clear patterns have yet emerged to support the prediction, Melzner et al. (2009) suggested that species exposed to naturally high and fluctuating CO_2 conditions may be pre-adapted to cope with increasing global oceanic CO_2 concentrations. The U.S. West Coast is naturally exposed to seasonally elevated CO_2 conditions of up to 1,000 µatm associated with wind-induced upwelling (Feeley et al. 2016) that may confer an advantage to speckled sanddab and other coastal species in the region. However, even among closely related species within this region, experiments have found varying levels of behavioral sensitivity to experimentally elevated CO_2 levels (Hamilton et al. 2014; Hamilton et al. 2017). Clearly more work is needed to understand the geographic and phylogenetic patterns driving this variation in sensitivity.

Alternatively, the observed resiliency of speckled sanddab may be related to the experimental approach or the specific set of behaviors examined in this study. The 4-6 week acclimation time to elevated CO_2 conditions used in this study is longer than that applied in many studies that have shown OA-induced behavioral disruptions (Kroeker et al. 2013). In most studies to date, routine (or "baseline") behavior and activity levels in the absence of specific food or predator stimuli have appeared to be minimally influenced by elevated CO_2 levels (e.g. Jutfelt and Hedgärde 2015, Sundin et al. 2017). Most of the studies showing the clearest effects of sensory-mediated behavioral change at elevated CO_2 levels have examined responses to predatory cues (e.g., Dixson et al. 2010, Welch et al. 2014), whereas sensitivity of foraging responses appears more variable among species (e.g., Cripps et al. 2011, Maneja et al. 2013, Dixson et al. 2014, Heinrich et al. 2016). Our behavioral characterization experiment suggested that the damaged skin cues functioned more like a foraging than predation cue in juvenile speckled sanddab. Hence, additional work should consider the effects of OA on responses to the visual predator cue or other aspects of behavior in juvenile speckled sanddab.

4.3 Conclusions

The elevated CO_2 levels associated with ongoing ocean acidification have been shown to alter aspects of behavior in a variety of marine species, but the effects are far from universal: significant variation in responses has been observed across species, ecological guilds, behavioral traits, and testing conditions. The observed resiliency of baseline activity patterns and foraging responses of speckled sanddab calls for continued examination of a diversity of species in temperate food webs. Speckled sanddab exhibited a counter-intuitive response to the damaged skin cues commonly applied in OA studies, demonstrating the need for caution in the interpretation of behavioral changes in species whose basic behavioral ecology has not been explored. Understanding the potential for OA-induced behavioral disruptions to impact marine ecosystems will require the merging of rigorous experimental protocols and a greater effort to understand the relevancy of impacted behaviors in the life history of the organism.

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Table 1. Conditions (mean ± SE) during acclimation and behavioral observations of speckled sanddab (*Citharichthys stigmaeus*) behavioral responses to elevated CO_2 levels.

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
$\Omega_{\text{Aragonite}}$	1.55 ± 0.03	0.81 ± 0.05	0.53 ± 0.03

Table 2. Primary statistics describing behavioral characterization experiment results. RM ANOVA was used to determine overall cue effects on behaviors for each period. Orthogonal linear comparisons were used to compare responses to potential predation cues to those of the SW control. Statistically significant (<, >) and non-significant trends (≤, ≥) within the "result" columns indicate the direction of the effect. ANCOVA was used to examine the effects of body size (*avgSL*) on behavioral responses within each period.

Overall effect of cue and period: RM ANOVA

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

Cue-specific results: Orthogonal linear comparisons to SW

	Cue Period		Feeding Period		Post-feeding Period	
Cue	*Result*	*p-value*	*Result*	*p-value*	*Result*	*p-value*
V		0.543	V < SW	0.047	V ≤ SW	0.191
DS		0.310	DS ≥ SW	0.176		0.572
PO		0.311		0.467		0.485

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
Feeding	Δp	0.008	0.427	0.050
	F_{min}	0.170	0.002	0.909
	F_s	0.051	0.004	0.615
Post-Feeding	Δp	0.290	0.711	0.889

Table 3. Primary statistics describing results of the OA experiment. RM ANOVA was used to determine the overall effect of CO_2 on behaviors for each stage. ANCOVA was used to examine the effects of body size (*avgSL*) on behavioral responses within each period of each stage. Survival analyses were used to determine the effect of CO_2 treatment on F_a and F_{min}.

Overall effect of CO_2 during each stage: RM ANOVA

			p-values		
Main Effects	RM	Stage	*CO_2*	*period*	*CO_2 x period*
CO_2	period	Baseline	0.882	< 0.001	0.444
		First-Feeding	0.437	< 0.001	0.609
		DS Response	0.631	< 0.001	0.722
		Post-DS Response	0.376	< 0.001	0.209

Table 3 Continued.

Size effects on responses: ANCOVA

Stage	Metric	Cue Period p-values			Feeding Period p-values			Post-Feeding Period p-values		
		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
	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
	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
	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
	F_{min}				0.608	0.850	0.050			
	F_s				0.349	0.163	0.663			

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

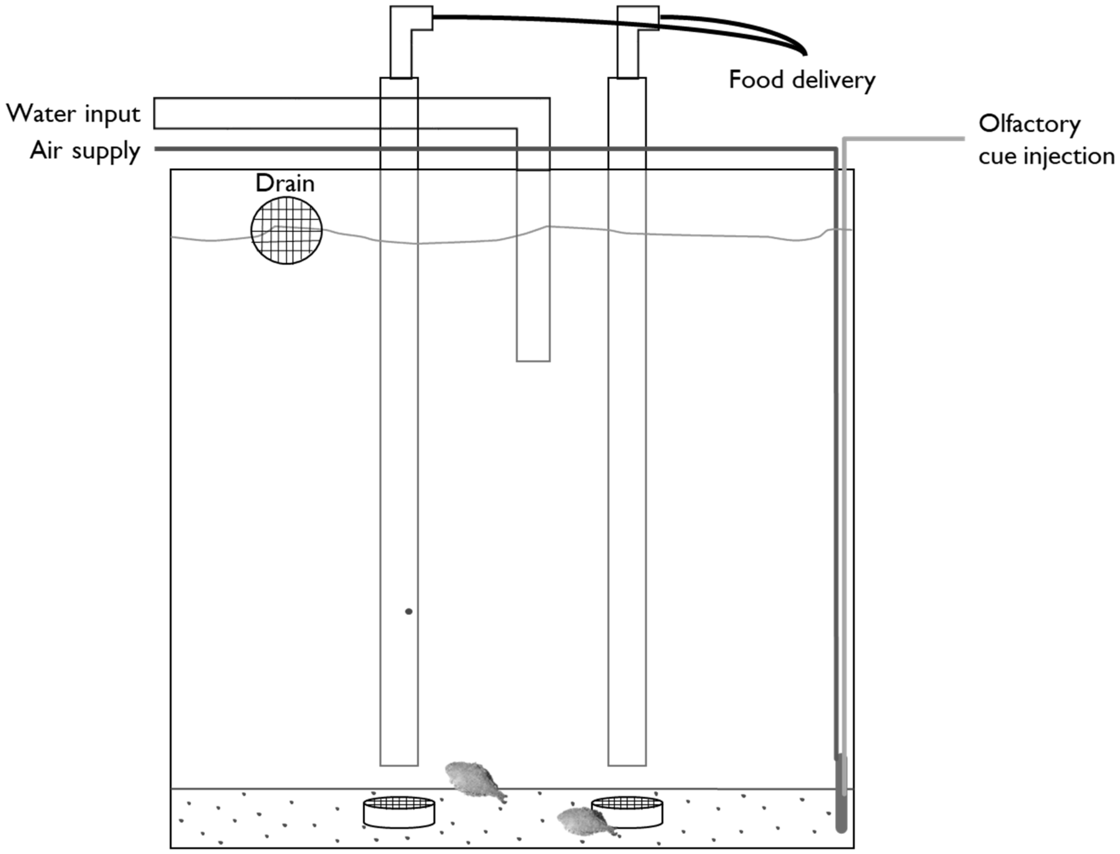

Fig. 1. Schematic of the experimental apparatus. Experiments took place in 125 L cubic flow-through tanks. Food was delivered through transparent, vertical tubes suspended 6 cm above a mesh grate. Flexible tubing delivered olfactory cues near the air stone to ensure adequate mixing of cues throughout the tank.

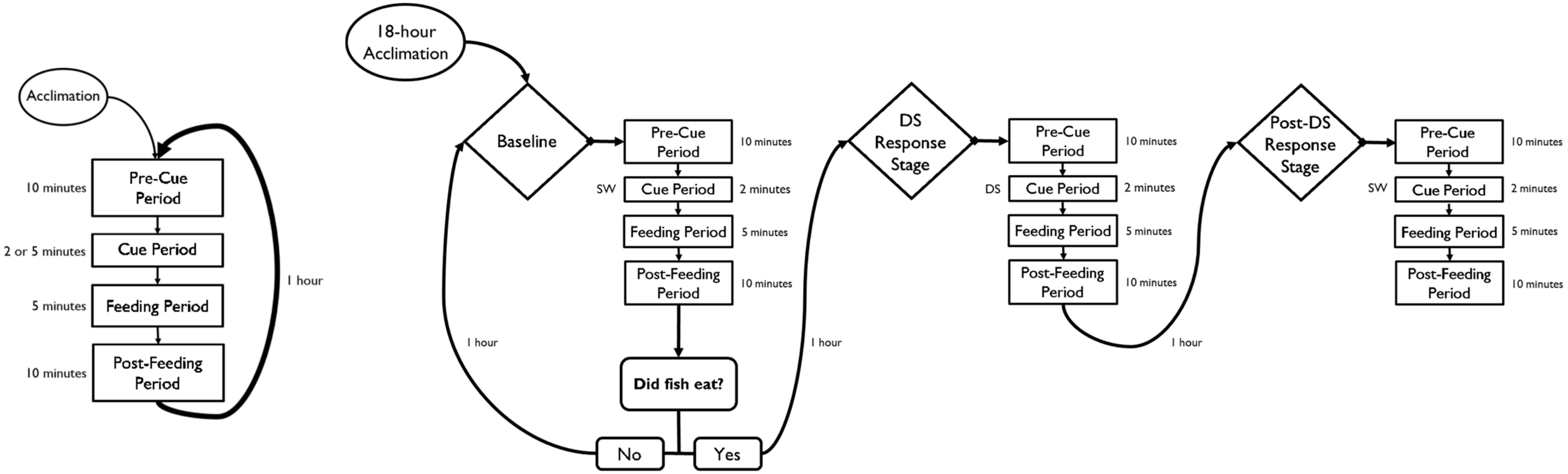

Fig. 2. Flow chart of experimental procedures for behavioral characterization experiment (left) and ocean acidification experiment (right). In the behavioral characterization experiment, potential predation cues were presented at 1-h intervals in a randomized order. In the OA experiment, trials consisted of three stages, each comprised of a cue presentation. Damaged skin cues (DS) were presented during the cue period of the DS response stage, while seawater (SW) functioned as a cue control for all other stages. If a pair of fish did not attempt to feed during the (I) baseline stage, this stage was repeated at 1-h intervals. Once a fish attempted to feed, it was subsequently tested in the following two stages.

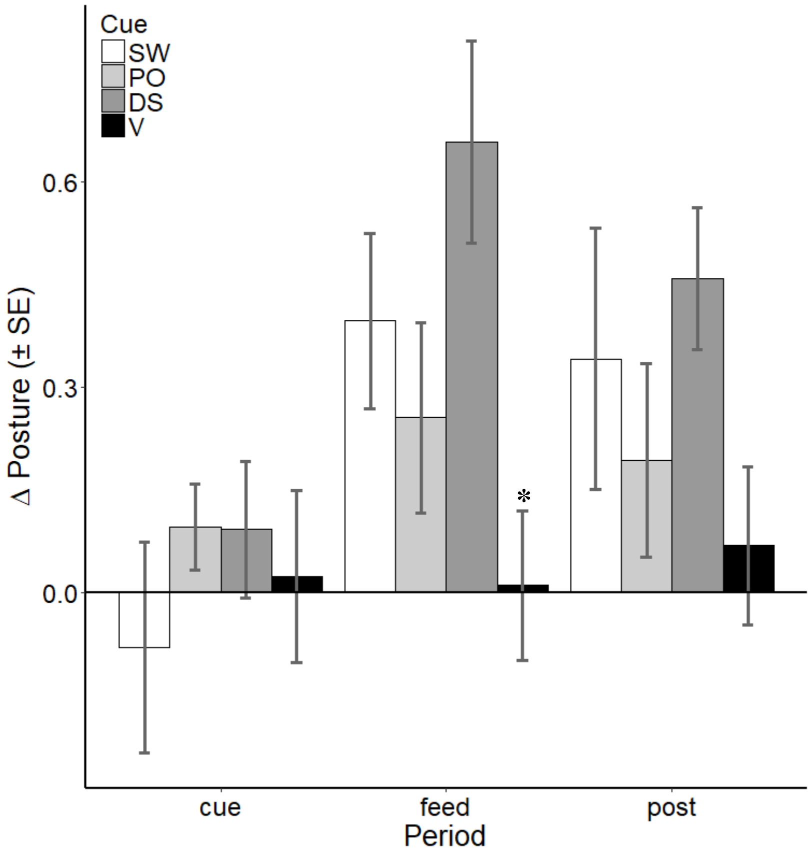

Fig. 3. Changes in speckled sanddab posture (mean ± SE) from initial pre-cue levels in subsequent (cue, feeding, and post-feeding) periods for each cue. Potential predation cues included predator odor (PO), damaged skin cues from injured conspecifics (DS), and the sight of a predator (V). A seawater (SW) blank was used as a cue control. Asterisks (*) indicate significant differences from response to SW.

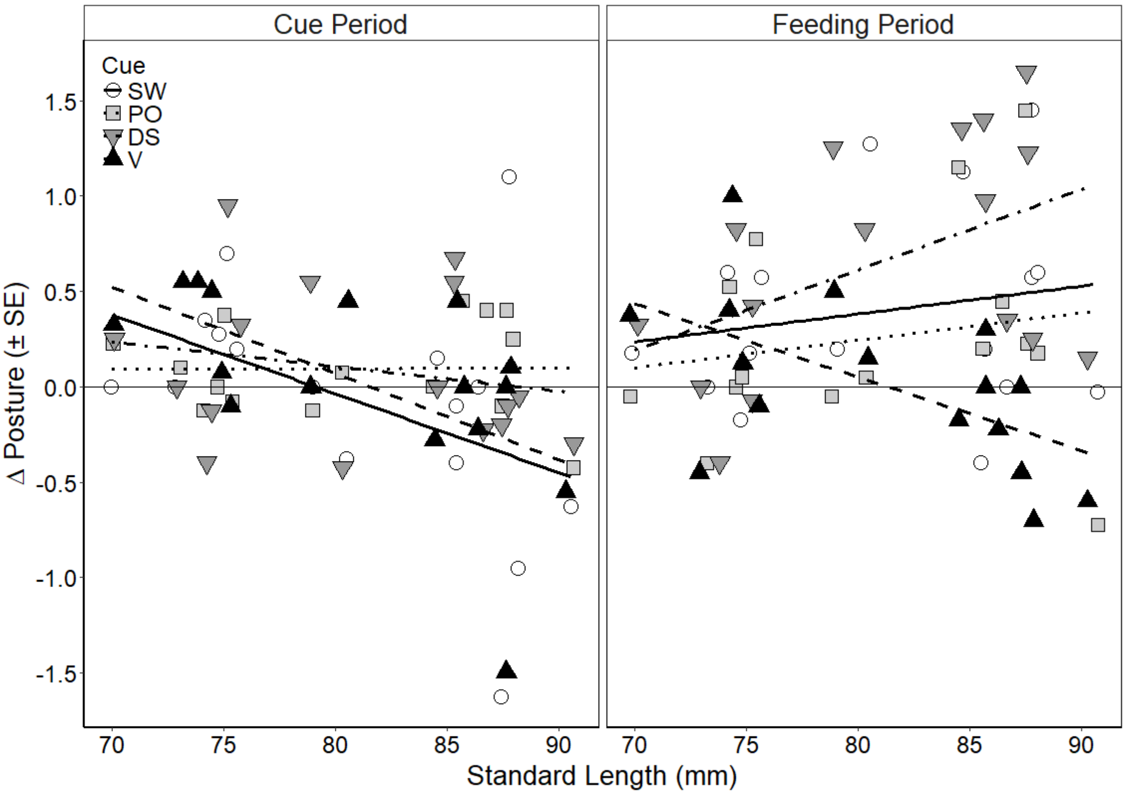

Fig. 4. Effect of fish size (mean size of experimental pair) and cue type on changes in posture from the pre-cue period to the cue period (left) and feeding period (right). Linear regressions between the mean size of each pair of sanddab and their behavioral responses are shown for each cue type of each trial. Note: overlapping points are slightly offset for clarity.

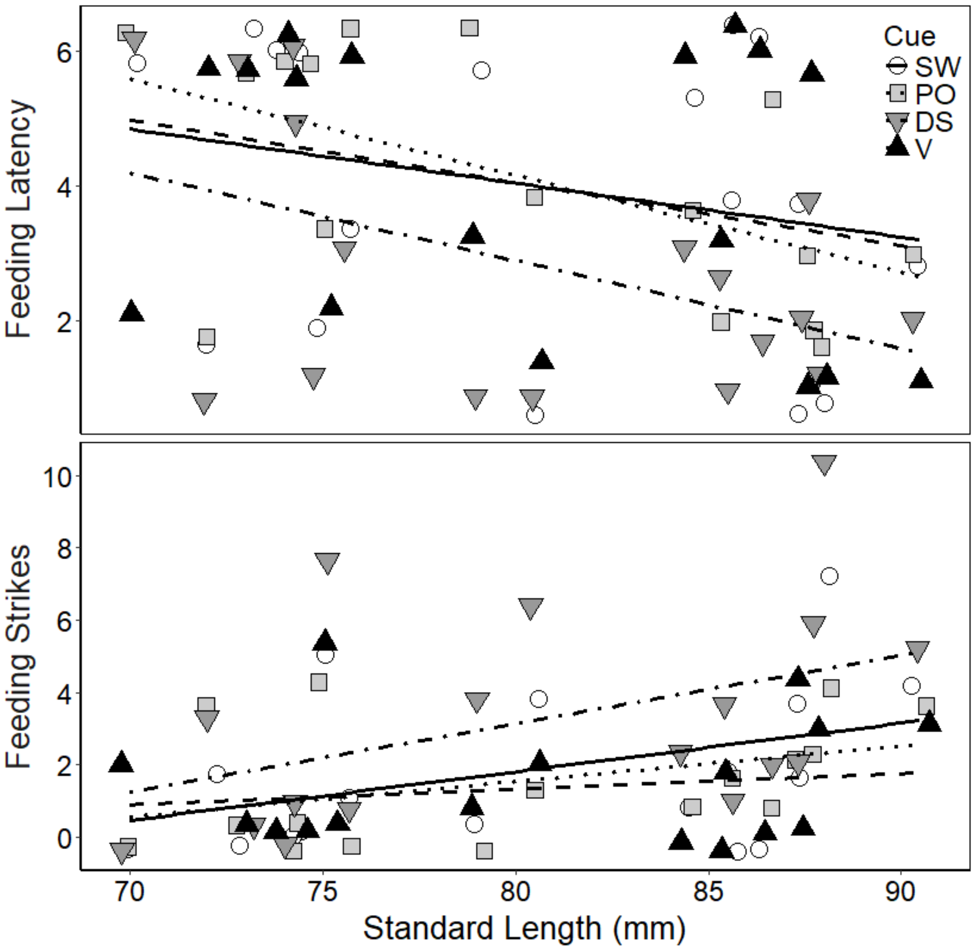

Fig. 5. Effect of fish size (mean size of experimental pair) and cue type on speckled sanddab feeding latency (top) and number of feeding strikes (bottom). Feeding latency was quantified as the minute within the feeding period (score of 1-5) in which one of the two fish first attempted to feed. Pairs that did not attempt to feed were assigned a score of 6. Note: overlapping points are slightly offset for clarity.

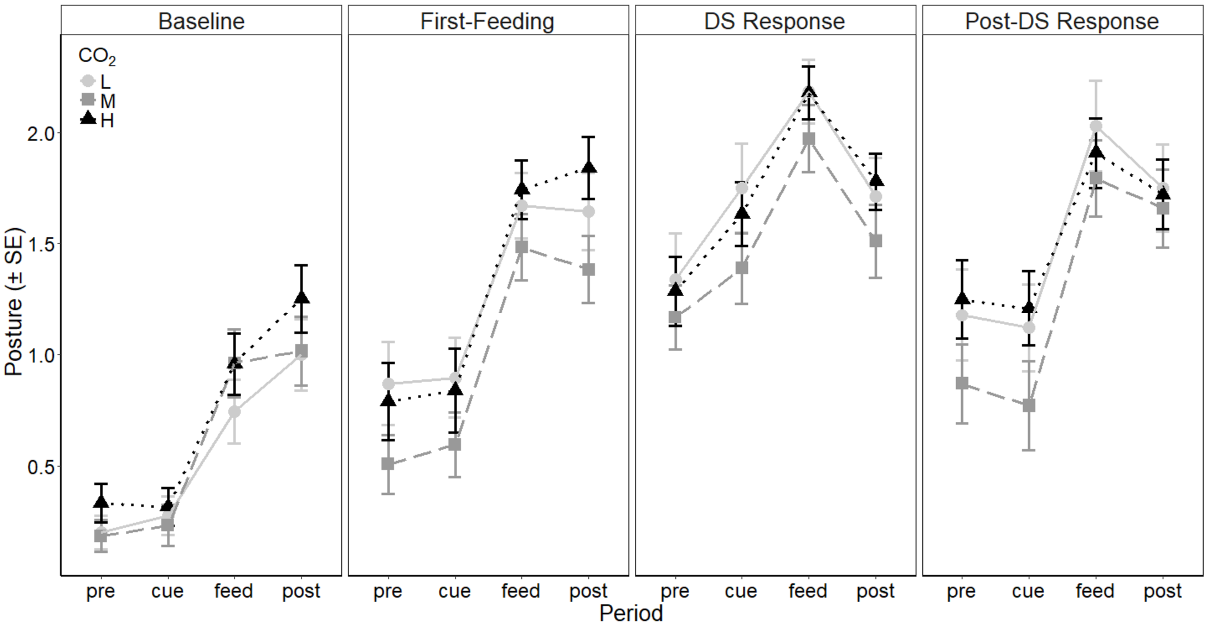

Fig. 6. Speckled sanddab posture (mean ± SE) during each period of the baseline, first-feeding, DS response, and post-DS response stages for each CO_2 treatment. Damaged skin (DS) cues were introduced during the cue period of the DS response stage. Seawater (SW) was introduced as a cue control during the cue periods of all other stages.

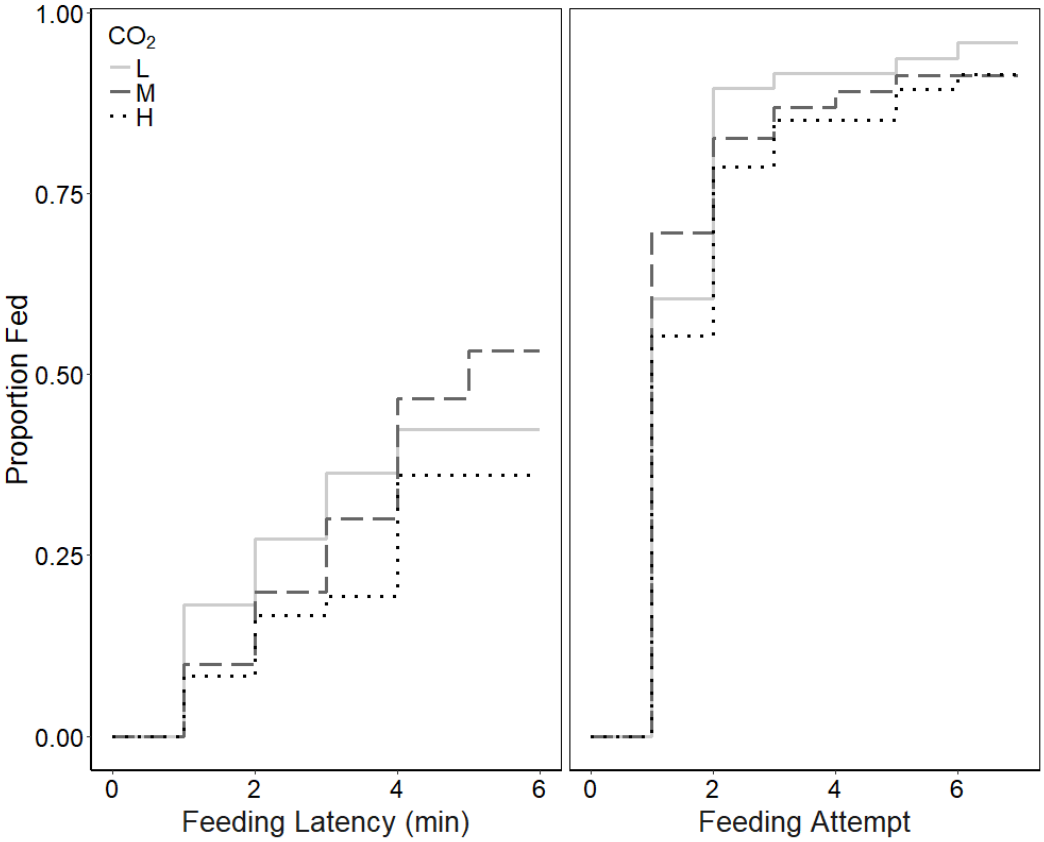

Fig. 7. Effect of CO_2 treatments on speckled sanddab willingness to feed; cumulative proportion of sanddab pairs feeding over time during the baseline stage (left) and during repeated testing of the baseline stage (right).

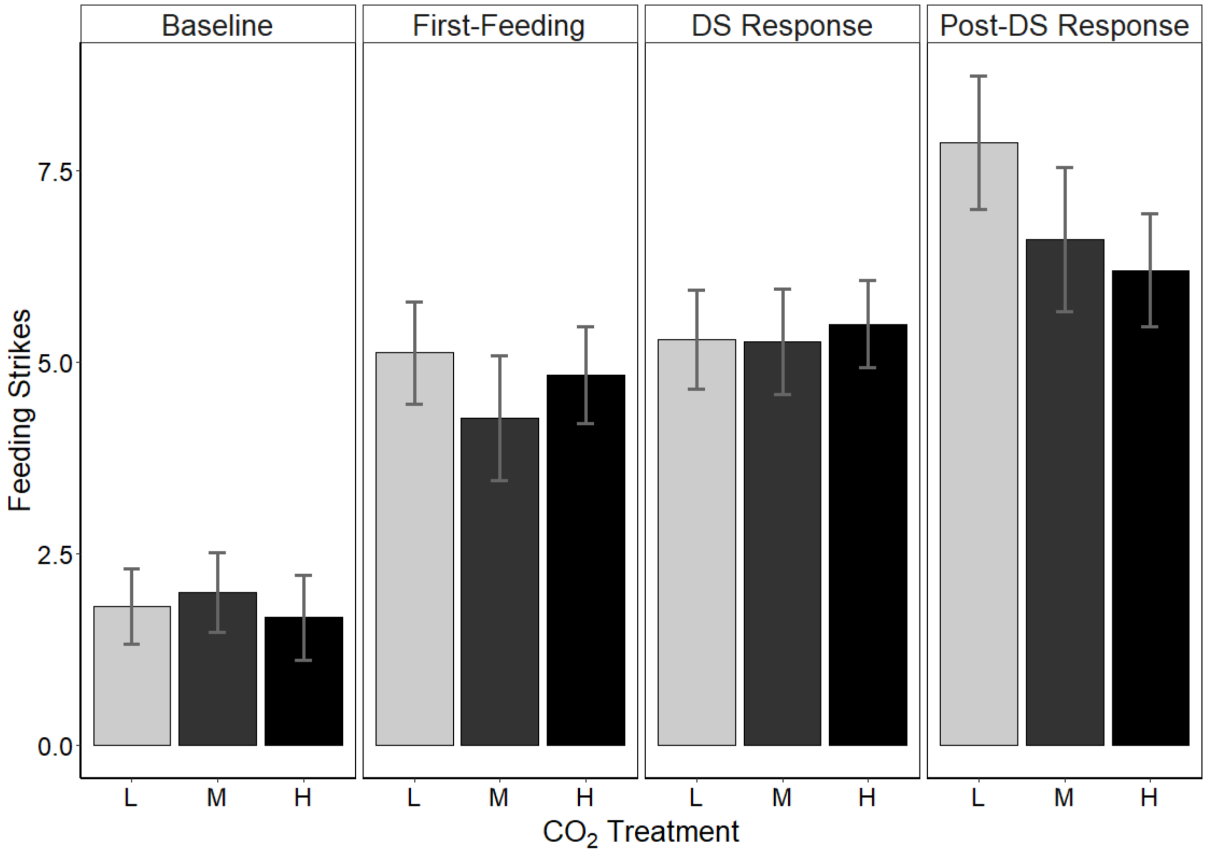

Fig. 8. Number of feeding strikes (mean ± SE) within each stage and CO_2 treatment.