Ocean acidification impairs olfactory discrimination and homing ability of a marine fish

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The persistence of most coastal marine species depends on larvae finding suitable adult habitat at the end of an offshore dispersive stage that can last weeks or months. We tested the effects that ocean acidification from elevated levels of atmospheric carbon dioxide (CO_2) could have on the ability of larvae to detect olfactory cues from adult habitats. Larval clownfish reared in control seawater (pH 8.15) discriminated between a range of cues that could help them locate reef habitat and suitable settlement sites. This discriminatory ability was disrupted when larvae were reared in conditions simulating CO_2-induced ocean acidification. Larvae became strongly attracted to olfactory stimuli they normally avoided when reared at levels of ocean pH that could occur *ca.* 2100 (pH 7.8) and they no longer responded to any olfactory cues when reared at pH levels (pH 7.6) that might be attained later next century on a business-as-usual carbon-dioxide emissions trajectory. If acidification continues unabated, the impairment of sensory ability will reduce population sustainability of many marine species, with potentially profound consequences for marine diversity.

climate change | larval sensory mechanisms | population connectivity | population replenishment

Ocean acidification caused by the uptake of additional carbon dioxide (CO_2) at the ocean surface is now recognized as a serious threat to marine ecosystems (1–4). At least 30% of the anthropogenic CO_2 released into the atmosphere in the past 200 years has been absorbed by the oceans, causing ocean pH to decline at a rate ≈100 times faster than at any time in the past 650,000 years (1, 4). Global ocean pH is estimated to have dropped by 0.1 units since preindustrial times and is projected to fall another 0.3–0.4 units by 2100 because of existing and future CO_2 emissions (1, 5–6). Considerable research effort has focused on predicting the impact that reduced carbonate-ion saturation states that accompany ocean acidification will have on calcifying marine organisms, particularly corals and other invertebrates that precipitate aragonite skeletons (2–3, 6). However, the effects that ocean acidification will have on other marine organisms, including fishes, remain almost completely unknown, especially for conditions of atmospheric carbon dioxide and seawater pH that could occur in the near future (4, 7–9).

The persistence of most coastal marine species depends on the ability of larvae to locate suitable settlement habitat at the end of a pelagic stage that can last weeks or months. Accumulating evidence for reef fishes suggests that both reef sounds (10) and olfactory cues (11–13) are used by larvae to locate reefs. The olfactory organs of many reef fishes are well-developed by the end of the larval phase (14–15), and it has recently been shown that larvae of some species can discriminate the smell of water from their natal reef compared with water from other reefs (13), which provides a mechanism to explain high levels of self-recruitment in some reef fish populations (16–19). It is well known that coral reef fish larvae can use olfactory cues to identify suitable settlement sites once they are in the vicinity of reef habitat. Settling larvae have been shown to respond to olfactory signals from preferred microhabitats (12, 20), resident conspecifics (21–23), or symbiotic partners such as anemones (24–25). Any disruption to the ability of larvae to detect and discriminate between olfactory cues that guide them to reefs, or that enable them to select preferred settlement habitat, would have far-reaching implications for the sustainability of adult populations.

We tested if elevated CO_2 and reduced seawater pH consistent with ocean acidification predictions could affect the ability of orange clownfish (*Amphiprion percula*; Pomacentridae. Fig. 1) larvae to respond to olfactory cues that are used to locate reef habitat and distinguish preferred settlement sites. Specifically, we tested the ability of settlement-stage larvae to respond to olfactory cues that are preferred during the settlement process compared with olfactory cues that are likely to be avoided when searching for reefs and settlement sites. Orange clownfish mostly live on oceanic reefs surrounding vegetated islands and recent research has shown that the larvae can discriminate between seawater from reefs surrounding vegetated islands versus seawater from reefs without islands (26). Furthermore, the larvae are positively attracted to water-borne cues from tropical rainforest trees (26) that should provide a reliable cue to the presence of vegetated oceanic islands. We tested the response of larval clownfish to olfactory cues from a range of tropical vegetation types when reared in seawater simulating 2 future CO_2-induced acidification scenarios (seawater pH 7.8 and 7.6) compared with current-day controls (pH 8.15). For larvae reared in each treatment we tested preference or avoidance of olfactory cues from the leaves of 3 vegetation types: (*i*) a tropical rainforest tree (*Xanthostemon chrysanthus*) that is a positive cue for settling clownfish (26), (*ii*) a swamp tree (*Melaleuca nervosa*) that contains pungent oils in the leaves and is avoided by settling clownfish (26), and (*iii*) a tropical savannah grass (*Megathyrsus maximus*) that is not expected to provide a reliable cue for the presence of trees on islands.

It is well known that anemonefishes are positively attracted to olfactory cues of host anemones (24–25). Therefore, we also tested the ability of larval clownfishes to respond to anemone olfactory cues when reared at the 3 pH levels. Finally, the presence of adult populations should be a good signal of favorable habitat and previous studies have found that larvae of some reef fishes are attracted to olfactory cues from conspecific adults (21–22). However, in species such as the orange clownfish where many larvae recruit to natal reefs (19), it would also be advantageous for juveniles to be able to discriminate between their parents and other adults to avoid inbreeding that could result

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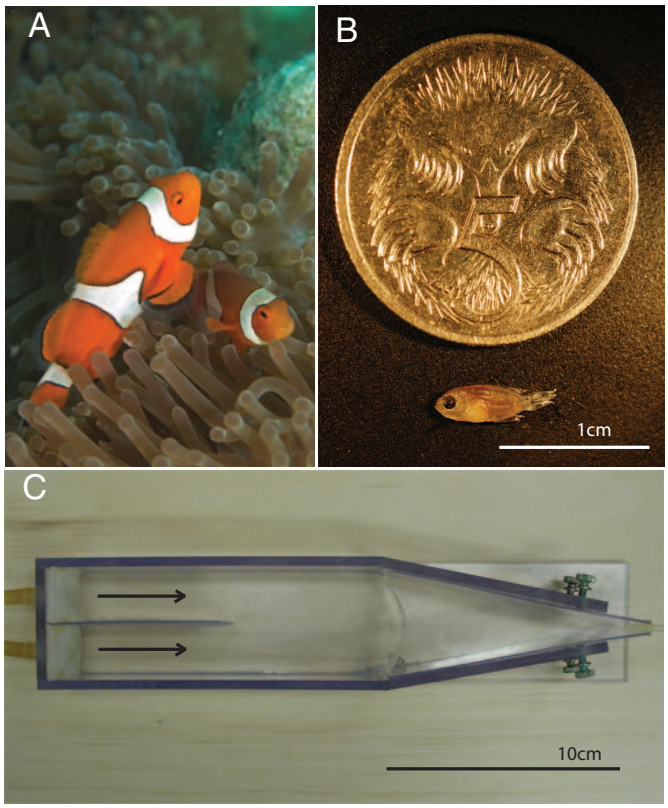

Fig. 1. Clownfish larvae use olfactory cues to locate adult habitat at the end of their pelagic stage. (*A*) Adult orange clownfish *A. percula* form breeding pairs on host anemones. (*B*) A settlement stage (11 days posthatching) larva of *A. percula* beside an Australian 5 cent coin for scale. (*C*) The Atema flume chamber used to test the responses of larvae to olfactory cues from leaves, anemones, and conspecifics in control and acidified water. Water from 2 different sources flowed through the chamber in the direction of the arrows. A larva was placed in the test section below the central partition and its position recorded at 5-s intervals for 2 min. The water sources were swapped and the procedure repeated.

from settling in their natal anemone. Therefore, we also tested the response of clownfish larvae to olfactory cues from their parents and other adult clownfish when reared at the 3 pH treatments.

Clownfish were reared at James Cook University's experimental aquarium facility where the pH of unmanipulated seawater was 8.15 ± 0.07. This is similar to the pH that pelagic larvae would experience during development in the open ocean (1). To simulate ocean acidification the pH of treatment seawater was adjusted to either 7.8 ± 0.05 or 7.6 ± 0.05 by the standard method of dissolving additional CO_2. The equivalent atmospheric CO_2 levels for the pH treatments in our experiment was estimated to be ≈1,000 ppm for pH 7.8 and ≈1,700 ppm for pH 7.6. These values are consistent with climate change models that predict atmospheric CO_2 levels could exceed 1,000 ppm by 2100 and approach 2,000 ppm by the end of next century under a business as usual scenario (5, 27). Clownfish were reared at 1 of the 3 pH levels from the day that eggs were laid until the larvae were competent to settle at 11 days after hatching. The olfactory responses of larvae were then tested in a 2-channel choice flume (13) where individuals were allowed to choose between a stream of seawater containing the olfactory cue to be tested and a stream of water without that cue.

Results

Larvae reared in control seawater spent equal amounts of time on each side of the chamber in a control test where neither

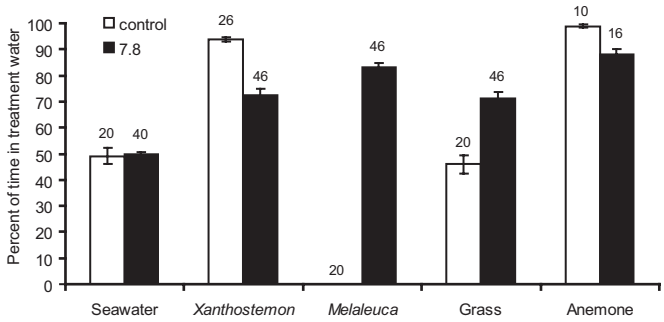

Fig. 2. Response of larval clownfish to olfactory cues from tropical plants and anemones when reared at current-day seawater pH (control, open bars) and in seawater, where the pH had been reduced using CO_2 to simulate the effect of ocean acidification (pH 7.8, filled bars). The first pair of columns shows the mean percentage of time that larvae spent of one side of a 2-channel flume chamber when neither stream of water in the chamber contained a test cue. Subsequent columns show the mean percentage of time that larvae spent in the stream of water containing the cue when one stream contained the cue and the other stream did not. Numbers above bars are the number of replicates for each test.

stream of seawater in the flume contained an additional olfactory cue (Fig. 2). Larvae exhibited a strong preference for *Xanthostemon* (Fig. 2, $P < 0.001$) spending >93% of their time in the stream of water in which leaves of this rainforest tree had been soaked. In contrast, all larvae completely avoiding the stream of water in which *Melaleuca* leaves had been soaked (Fig. 2, $P < 0.001$). Avoidance of *Melaleuca* might be expected because the leaves of these trees contain pungent oils. Larvae showed no preference or avoidance for olfactory cues from grass, spending approximately equal amounts of time in the stream of water in which grass leaves had been soaked and in the stream of water without olfactory cues from grass (Fig. 2, $P > 0.1$). As expected, larvae also exhibited a strong preference for anemones (Fig. 2, $P < 0.001$), spending nearly all of their time in the stream of water in which an anemone had been placed for 2 h.

Larvae reared in seawater at pH 7.8 exhibited significant differences in olfactory responses to larvae reared in control seawater for all comparisons. Although the preferences for *Xanthostemon* and anemones remained, there was a significant reduction in the strength of the response compared with controls (Fig. 2; $P < 0.001$ and $P < 0.01$ respectively). Larvae exhibited a preference for grass that did not occur in larvae reared in control water (Fig. 2, $P < 0.001$), and, more dramatically, larvae now exhibited a strong preference for olfactory cues from *Melaleuca* (Fig. 2, $P < 0.001$). Larvae reared in seawater at pH 7.8 spent >80% of their time in the stream of water in which *Melaleuca* leaves had been soaked, even though larvae reared in control water completely avoided this cues. Larvae still spent an equal amount of time on each side of the chamber in a control test where neither stream of seawater in the flume contained an additional olfactory cue (Fig. 2).

Larvae reared in control seawater almost completely avoided the water stream containing olfactory cues from their own parents (Fig. 3, $P < 0.001$), but exhibited a strong preferences for a water stream containing cues from other adults (Fig. 3, $P < 0.001$). The ability to discriminate between parents and other adults was lost in larvae reared at pH 7.8. These larvae exhibited an equally strong preference for olfactory cues from their own parents ($P < 0.001$) and other adult orange clownfish (Fig. 3, $P < 0.001$). These responses were confirmed when larvae were presented simultaneously with cues from their own parents and other adults. Larvae reared in control water continued to prefer the water stream with

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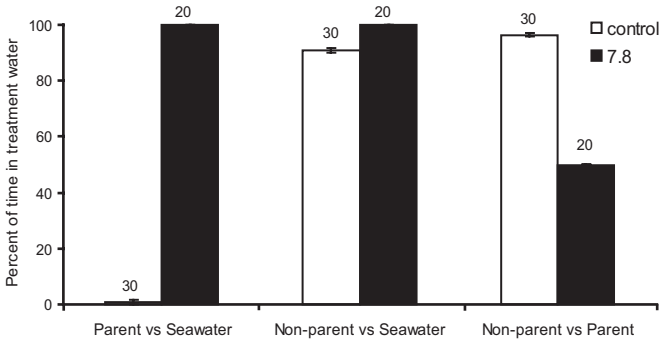

Fig. 3. Response of larval clownfish to olfactory cues from their parents and other adult clownfishes when reared at current-day seawater pH (control, open bars) and in seawater the pH had been reduced using CO_2 to simulate the effect of ocean acidification (pH 7.8, filled bars). The first 2 pairs of columns show the mean percentage of time that larvae spent in the stream of water containing the cue when one stream contained the cue and the other did not. The third pair of columns shows the mean percentage of time that larvae spent in the stream of water containing olfactory cues from non-parents when one stream contained olfactory cues from non-parents and the other contained olfactory cues from their parents. Numbers above bars are the number of replicates for each test.

olfactory cues from other adults (Fig. 3, $P < 0.001$), whereas larvae reared in pH 7.8 water showed no preference ($P > 0.1$), spending equal time in each water stream (Fig. 3).

Larvae reared in pH 7.6 seawater did not respond to any of the olfactory cues presented in the flume chamber, either for anemones, plants, conspecifics or parents. Larvae remained passively at the end of the chamber and did not respond to the presence of a water stream containing an olfactory cue, or to reversal of the stream of water containing the cue. Larvae reared in control pH but tested in pH 7.6 water exhibited the same choices as larvae reared and tested in control water ($P > 0.1$ for all comparisons). Similarly, larvae reared at pH 7.6 but tested in control water did not respond to any olfactory stimuli, just as larvae reared in tested in pH 7.6 water did not respond to any olfactory cues presented. This shows that extended exposure to low pH water affected the olfactory system and that the observed changes were not caused by an immediate interference to the fishes' olfactory capability or chemical modification of the odor signal. Further studies are needed to determine if the effects on the olfactory system are reversible.

Larvae reared at pH 7.6 and pH 7.8 had the same morphological appearance as larvae reared in control water, swam and fed normally in the rearing tanks, and exhibited similar settlement behavior (positive attraction to the wall of the rearing tank) at day 11, indicating that they were not developmentally retarded. Detailed examination of the nasal cavity of 3 larvae from each pH treatment using scanning electron microscopy revealed no apparent difference in the development of the external olfactory system that might be responsible for the different behavioral responses observed among treatments. All larvae had a well-developed sensory epithelium with a dense covering of cillia and numerous olfactory receptor neurons.

Discussion

Our results show that elevated CO_2 and reduced seawater pH that could occur early next century in the world's oceans can dramatically affect the behavioral decisions of marine organisms during critical stages of their life-history. In this case, acidification disrupted the olfactory mechanism by which clownfish larvae discriminate between cues that may be useful for locating suitable adult habitat and other cues that could lead larvae to unsuitable settlement sites. Attraction to olfactory signals that are normally avoided, in combination with a reduced preference for favorable cues, could clearly cause fish larvae to be attracted to locations that are suboptimal for settlement or are devoid of settlement habitat. Olfactory cues appear to be a key mechanism by which the pelagic larvae of many coastal marine species identify and navigate toward adult habitat and then select suitable settlement sites (11–13, 15, 21–26, 28). Disruption to this process would have significant consequences for the replenishment of adult populations and could lead to the decline of many coastal species.

Recent research has found that a significant proportion of larval from some reef fish populations return to their natal reef (16–19) and that the ability to discriminate olfactory cues of natal reefs appears to be an important mechanism mediating this behavior (13, 26, 28). Reduced ability to respond to the cues from natal reefs would cause changes to patterns of dispersal and connectivity in these populations, because fewer larvae would be retained locally. Changes in connectivity patterns, along with a general reduction in the total number of individuals recruiting to populations because of impairment of the habitat selection process, would have consequences for the efficacy of marine protected areas and sustainability of reef fisheries (19, 29). Even if larvae do return to their natal reefs, our results show that population would be more prone to inbreeding because larvae are no longer able to discriminate between parents and nonparents when choosing settlement sites.

Settlement stage larvae of many reef fish species have well developed olfactory systems (14–15), which is indicative of the importance of olfaction in locating suitable adult habitat at the end of the pelagic larval stage. Electron microscopy did not reveal any differences in the internal morphology of the nasal organ of larval clownfishes that might be responsible for the different behavioral responses to olfactory stimuli exhibited among treatments. This suggests that elevated CO_2 and low pH are disrupting the transfer of chemosensory signals within the neurosensory system, not affecting the development of the external sensory apparatus. Both CO_2 and pH covaried in our experiment, therefore, it is not possible to determine if the effects on larval behavior we observed were due to the direct effect of reduced seawater pH on the olfactory system, the effects of elevated CO_2 on tissue pH (7), or a combination of both. Nevertheless, the CO_2 and pH levels were within the range of values predicted for *ca.* 2100 (≈1,000 ppm CO_2 and pH 7.8) and near the end of next century (≈1,700 ppm CO_2 and 7.6) under a business-as-usual scenario for CO_2 emissions (5, 27). Further research is required to establish the precise physiological mechanisms responsible for dysfunctional olfactory capacity of larvae at elevated CO_2 and reduced pH.

The potential for acclimation or adaptation are important considerations when assessing the vulnerability of organisms to climate change (4, 8, 9, 30). The potential for most marine organisms to adapt to a rapid reduction in ocean pH has not been tested. However, ocean pH has changed little over the past 650,000 years (1–4), therefore, it is unlikely that many marine species possess genetic variation adapted to lower pH. Importantly, anthropogenically-induced ocean acidification is causing pH to decline >100 times faster than at any time in the past 650,000 years (1–4) and it is unlikely that genetic adaptation by most marine organisms, perhaps except those with very rapid generation times, will be able to adapt to keep pace with such a rapid rate of change (2).

In conclusion, we show that ocean acidification can affect the behavior of a marine organism during a critical life history stage. A loss of larval olfactory capacity in marine organisms through acidification could have significant consequences for marine biodiversity. Larvae of many marine species appear to navigate and orient toward suitable adult habitat and settlement substrata using a complex range of olfactory stimuli (28). If the olfactory

system is impaired at high CO_2 and low pH, this important life history transition will be disrupted and populations will not be properly replenished. This link between marine teleost olfaction and CO_2-induced reduction in seawater pH strengthens the case for substantial cuts to CO_2 emissions to stabilize the atmospheric concentration and limit the extent of future ocean acidification.

Materials and Methods

Larval Rearing. Clownfish were reared in a 70,000-L recirculating seawater system at James Cook University's experimental marine aquarium facility. Larvae were offspring of 14 breeding pairs kept in separate 70-L aquariums. Breeding pairs laid eggs on the underside of a terracotta pot placed in their aquarium. For each breeding pair, on the night of hatching (6–8 days) the pot was removed from the parental aquarium and transferred to a 100-L larval rearing aquarium. The appearance of the embryos identified their readiness to hatch. Larvae were reared in a semiclosed system, where each aerated aquarium had no water flow during the day and was then slowly flushed with filtered seawater each night. This daily cycle ensured that larvae could feed ad libitum throughout daylight hours and that any unconsumed food was removed each night. Larvae were fed rotifers (*Brachionus* sp.) at 5 individuals mL^{-1} each morning for the first 3 days. *Artemia* naupli were added at 1 individual mL^{-1} each morning from day 3. The ratio of *Artemia* naupli to rotifers was increased each day until larvae were only fed 5 *Artemia* naupli mL^{-1} from 8 days after hatching. A summer light cycle of 13 h of light/11 h of dark was simulated with fluorescent lights.

Seawater Manipulation. Seawater pH was adjusted by the standard method of dissolving carbon dioxide (CO_2) into water (31–33). A separate pH-controller (Tunze Aquarientechnik) was attached to each aquarium to maintain pH at the desired level (7.8 or 7.6) by CO_2 injection. The pH controller was connected to a laboratory-grade glass pH probe in the aquarium and to an electronic solenoid connected to a cylinder of CO_2. The solenoid injected a slow stream of CO_2 into a diffuser (Red Sea Reactor 500) at the bottom of the aquarium whenever the pH of the aquarium seawater rose above the set point. A precision needle valve inserted before the solenoid was adjusted to ensure a slow, steady, delivery of CO_2 into the diffuser. The diffuser also served as a vigorous stirrer. Using this method is was possible to constantly maintain pH within $\pm$ 0.05 units of the desired level and there was no detectable gradient in seawater pH within the aquarium. The equivalent atmospheric concentration of CO_2 for each pH treatment was estimated by sealing replicate tanks ($n = 6$) in which the pH had been adjusted by CO_2 injection and then measuring the increase in pCO_2 in a narrow headspace above the water surface with a Vaisala GM70 CO_2 meter (Vaisala, Finland). The respective increase in atmospheric CO_2 above ambient levels was estimated to be 660 $\pm$ 23 ppm for the pH 7.8 treatment and 1,323 $\pm$ 55 ppm for the pH 7.6 treatment. This equates to atmospheric CO_2 levels of approximately1,050 ppm for pH 7.8 and 1,710 ppm for pH 7.6.

The pH of each aquarium was independently validated 4 times per day using a WP80 pH meter (TPS) calibrated daily with fresh pH buffers (Merk). CO_2 was only injected to adjust pH when eggs or larvae were present. All water returned to a 60,000-L sump where it was degassed by stirring, filtered, and any additional nutrients removed in a 1,000-L algal bio-remediation tank. Partial exchanges with freshly collected seawater occurred weekly. Water temperature was maintained at 30 °C $\pm$ 0.6 (SD) in the breeding and rearing aquariums using electric heaters. Oxygen levels were maintained above 90% saturation by the mixing action of the diffuser pump. Oxygen levels were measured 4 times daily with a WTW Oxi 340i oxygen probe.

Olfactory Discrimination Trials. *A. percula* have a pelagic larval duration of $\approx$11–12 days (19). Behavior consistent with competency to settle (attraction to the sides of the rearing aquarium) and the appearance of benthic coloration (a white stripe on the gill cover) occurred at 11 days posthatching. The response of 11-day-old larvae to olfactory cues was tested in a 2-channel choice flume (13 cm $\times$ 4 cm) (13). Larvae were released at the downstream end of the flume where they were free to move to either side of the chamber. A constant gravity-driven flow of 100 mL min^{-1} per channel was maintained throughout all trials. Flow rates were measured using a flow meter and dye tests were conducted at each water change to ensure that the 2 channels exhibited distinct and parallel water flow, with no turbulence or eddies. For each trial, a single fish was placed into the center of the downstream end of the choice flume and acclimated to the 2 water choices for 5 min. At the end of the acclimation period, the position of the fish on each side of the chamber was recorded at 5-s intervals for a 2-min period. This was followed by a 1-min rest period, during which the water sources were switched, providing a control for potential side preferences that were not associated with the water source. After the switch of water sources, the entire test including the acclimation period, was repeated and the total time juveniles were associated with each water source was recorded. Randomly selected larvae from at least 3 different parents were tested for each combination of olfactory cue and pH. Each fish was tested only once.

In each trial, a larva was given a choice in the flume chamber between a water source treated with a specific olfactory cue and an identical water source without that cue. Artificial seawater (Red Sea brand) was used in the olfactory tests because it was assumed to contain no biological cues that the larvae may have become accustomed to during rearing. To prepare water to test the response of larvae to olfactory cues from each of the 3 species of terrestrial plants (*X. chrysanthus*, Myrtaceae; *M. nervosa*, Myrtaceae; and *M. maximus*, Poaceae) 20 g of leaves were cut into 1-cm squares and soaked in 10 L of artificial seawater for 2 h and then removed before the trials began. To test the response of larvae to olfactory cues from a host anemone, a single anemone was soaked in 10-L of artificial seawater for 2 h. To test the response of larvae to olfactory cues from their parents versus other adults, 10-L of water was taken directly from the parental tank, or from the tank of another adult pair.

Analysis. Kolmogorov–Smirnov tests were used to: (*i*) compare the proportion of time that individuals spent in the stream of water containing the olfactory cue in any given set of trials versus the proportion of time that individuals spent on one side of the chamber when there was no cue presented in either water stream (i.e., a null distribution for testing choices against) and (*ii*) compare the proportion of time that individuals spent in the stream of water containing the olfactory cue at the control pH versus the proportion of time that individuals spent in that stream of water at the treatment pH.

Electron Microscopy. Larvae selected for scanning electron microscopy were fixed and stored in 4% glutaraldehyde. Samples were washed in phosphate buffer (pH 7.34), dehydrated with ethanol and placed in acetone, they were then critical point dried, glued on a metallic table, and coated with a mixture of gold-palladium. Preparations were examined on a Jeol-JSM-6380LA scanning electron microscope.

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